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Applicants:	Andrew VAILLANT et al.
Serial Number:	10/661,097
Filing Date:	September 12, 2003
For:	ANTIVIRAL OLIGONUCLEOTIDES TARGETING HSV AND CMV
Art Unit:	1648
Examiner:	Jane J. ZARA
Agent:	Patrice Préville

DECLARATION UNDER 37 C.F.R. SEC. 1.132

I, Jean-Marc Juteau, do hereby declare and state as follows:

1. I received the degrees of Bachelor (B.Sc.) of Biology from Montreal University in 1985, Master (M.Sc.) of Microbiology and Immunology from Montreal University in 1988, and Doctor of Philosophy (Ph.D.) of Microbiology and Immunology from Laval University in 1991.
2. My academic background and experiences in the field of the present invention are listed on the enclosed *curriculum vitae*.
3. I am a founder since 1999 of REPLICor Inc. and Senior Vice President since 2002.
4. I am an author of several scholarly publications as listed in my enclosed *curriculum vitae*.

5. I am an inventor in the present application; I have read and am thoroughly familiar with the contents of U.S. Patent Application Serial No. 10/661,097 entitled "ANTIVIRAL OLIGONUCLEOTIDES TARGETING HSV AND CMV", including the claims.

6. I have also read and understood the latest Official Action from the PTO dated April 5, 2006. In this Office Action, claims 1-2 and 14-32 were rejected for lack of enablement under 35 U.S.C. §112, first paragraph.

7. The following experiments has been performed in April 2005 for herpes virus-2) and in Dec. 2005 for cytomegalovirus under the supervision of Andrew Vaillant (inventor on this invention) and myself, to obtain results with two animal models showing the *in vivo* antiviral activity occurring by a non-complementary mode of action of oligonucleotides (ONs) of the present invention. These *in vivo* models of viral infection are recognized models that can be used for the demonstration of a drug treatment activity (Cone *et al*, 2006, BMC Infect. Dis., 6:90; Bernstein *et al.*, 2003, Antimicrob. Agent Chemother., 47: 3784; Scott *et al.*, 2005, J. Gen. Virol., 86: 2141). These *in vivo* treatment models cover different viruses, different pathogenic aspects, different routes of administration, different drug dosages, different formulations and different oligonucleotides.

The following experiments were conducted to evaluate the therapeutic antiviral activity of sequence independent oligonucleotides *in vivo*.

a) **Herpes virus-2**

The vaginal human herpes simplex-2 (HSV-2) transmission model in mouse has been demonstrated to be useful in predicting clinical efficacy of topical microbicides against the transmission of HSV-2 (genital herpes).

The antiviral activity of ONs was tested in a topical (vaginal) model of transmission of HSV-2. A phosphorothioated 40randomer (REP 2006) or a phosphorothioated 40mer polyC (REP 2031) were applied topically as a single application in the vagina before vaginal infection with the virus. The sodium salts of REP 2006 or REP 2031 were dissolved in phosphate buffer saline (PBS) to 100mg/ml, heated to 65°C for 15 min then cooled to room temperature. Cooled solutions were then filter sterilized with a 0.22um cellulose acetate filter. Solutions were stored frozen at -20°C until use. Before application, samples were warmed to 37°C for 5 min. Antiviral activity was monitored by viral titration in the vagina (Table 1).

Table 1
ONs are effective topical agents against HSV-2 transmission

PS-ON dose	% of animals protected from transmission
0 (placebo control)	0/12
100mg/ml REP 2006	8/12
100mg/ml REP 2031	12/12

This data shows that ONs of this invention are effective antiviral agents in treating *in vivo* HSV-2 infection. In this *in vivo* model, administration of the compound was well tolerated.

b) Cytomegalovirus

The mouse model of cytomegalovirus (CMV) infection is a well established model for the study of systemic and organ, such as liver and spleen, infection. It has been used for testing activity of several treatments.

The activity of ONs was tested in an animal model of human CMV. A phosphorothioated 40 randomer (REP 2006) or phosphorothioated 40mer polyC (REP 2031) or a phosphorothioated-2'O-methyl 40randomer (REP 2107) were administered parenterally (as a daily 1ml intraperitoneal injection) for 2 days prior to infection, during infection and for 3 days after infection. The sodium salts of REP 2006, REP 2031 or REP 2107 were dissolved in normal saline (0.9% NaCl) to a concentration of 0.5mg/ml. Solutions were then heated to 65°C for 15 minutes, cooled to room temperature and filter sterilized using a 0.22um cellulose acetate filter. Sterile solutions were stored frozen at -20 deg C and thawed and warmed to 37°C prior to administration Antiviral activity was monitored by viral titration in the liver (Table 2).

Table 2
ONs are effective agents against systemic CMV infection

ON dose	Liver titer (log10/ml tissue)
0 (placebo control)	2.9
20mg/kg/day REP 2006	1.1
20mg/kg/day REP 2031	0.7
20mg/kg/day REP 2107	0.9

These results show that ONs of this invention are effective antiviral agents in treating *in vivo* CMV. In this *in vivo* model, administration of the compound was well tolerated.

8. The results presented hereinabove and produced according to the teaching disclosed in the U.S. Patent Application Serial No 10/661,097 clearly proves that the present invention has clinical relevance and in addition, that the *in vitro* results disclosed in the present application do not diverge from *in vivo* responses. The antiviral activity of the sequence independent oligonucleotides of the present invention is demonstrated in two different *in vivo* models of viral infections.

9. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by a fine or imprisonment, or both (18 U.S.C. Sec. 1001), and may jeopardize the validity of the application of any patent issuing thereon.

Signed  Dated: Oct. 1, 2006

Jean-Marc Juteau

Curriculum vitae

JEAN-MARC JUTEAU, Ph.D

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EXPERIENCE

01-2002 - today

Senior Vice-President and Founder, REPLICor Inc., Laval.
Biopharmaceutical company developing antiviral and anticancer drugs.

Responsibilities:

- Science development.
  Day to day contact with CSO, scientific input.
- In charge of intellectual property portfolio.
  Patent writing, strategy, management.

02-1999 – 01-2002

CEO and founder, REPLICor Inc., Laval.

Responsibilities:

- Science development
- In charge of financing
  Instrumental in raising $2.5M in equity and loan
- In charge of licensing and contract agreement
  Negotiation of licenses and contracts with universities

02-1996 to 02-1999

Officer, Office of Technology Transfer, McGill University, Montreal.

Responsibilities:

- Agreement management and negotiation
  License, research, option, confidentiality, material distribution.
- Spin-off company projects
  Set-up of spin-off company, contact with investors, business plan.

03-94 to 02-96

Product Manager, Iso Tech Design, Laval
Company developing and marketing micro-environments for pharma applications.

Responsibilities:

- Microbiology quality control..

- Distributor formation
  Contacts: Baxter Health Care, VWR, Khulman Tech., E.S.I. FluFrance, Liberty Clean Rooms, Millipore.

91 à 10-93
Director and Co-founder, DIAGNOGENE inc., R&D in biotechnology, Ste-Foy
Responsibilities: Financial and research administration, representation.

RESEARCH TRAINING

09-92 à 11-93
Post-doctoral scientist, **INRS-santé**, Pointe-Claire
Project: In-vitro mutagenesis of 4-chlorobenzoate dehalogenase in *Pseudomonas sp.* CBS3.

08-91 à 09-92
Post-doctoral scientist, **Institut de Recherches Cliniques de Montréal**
Project: Cloning et characterization of a cardiac specific transcription factor.

11-90
Training in molecular modeling, Department of Molecular and Cell Biology, **University of Connecticut.**

05-88 to 06-88
Workshop on DNA technologies: Sequence and in-vitro mutagenesis, **University of North-Carolina**, Chapel Hill, NC.

EDUCATION

87-91
Doctorate (Ph.D)., Microbiology and Immunology, **Laval University.**
Molecular biology, epidemiology and structure-function analysis of the ROB-1 ß-lactamase.

85-87
Master (M.Sc)., Microbiology and Immunology, **Montreal University and Hôtel-Dieu Hospital.**
Granulocytar function in recurrent vaginitis.

82-85
Bachelor (B.Sc.)., Biology, **Montreal University.**

BOARD MEMBERSHIP

2005- today
Member of the Montreal Life Science Committee.

2004- today
President of the Alumni Association of Montreal Clinical Research Institute.

SCHOLARSHIP, AWARD and PRIZES

Industrial Design Prize 1995 from the Design Institute (received in team for a micro-environment)
Institut National de la Recherche Scientifique (INRS) Fellowship, 1992-93.
Medical Research Council (MRC)Fellowship, 1992.
Fonds de la Recherche en Santé du Québec (FRSQ) Studentship, 1989-90-91.
Fonds pour la Formation des Chercheurs et l'Aide à la Recherche (FCAR) Studentship, 1988-89.
Canlab Prize from l'Association des Microbiologistes du Québec, 1989.

AUTHORSHIP

Patent filings:	20
Scientific articles:	10
Posters and oral presentations:	30

Vaillant A, Juteau JM, Lu H, Liu S, Lackman-Smith C, Ptak R, Jiang S. Phosphorothioate oligonucleotides inhibit human immunodeficiency virus type 1 fusion by blocking gp41 core formation. Antimicrob Agents Chemother. 2006 Apr;50(4):1393-401.

Kocisko DA, Vaillant A, Lee KS, Arnold KM, Bertholet N, Race RE, Olsen EA, Juteau JM, Caughey B. Potent antiscrapie activities of degenerate phosphorothioate oligonucleotides. Antimicrob Agents Chemother. 2006 Mar;50(3):1034-44.

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Ahmad D, Fraser J, Sylvestre M, Larose A, Khan A, Bergeron J, Juteau JM, Sondossi M. Sequence of the bphD gene encoding 2-hydroxy-6-oxo-(phenyl/chlorophenyl)hexa-2,4-dienoic acid (HOP/cPDA) hydrolase involved in the biphenyl/polychlorinated biphenyl degradation pathway in Comamonas testosteroni: evidence suggesting involvement of Ser112 in catalytic activity.Gene. 1995 Apr 14;156(1):69-74.

Juteau JM, Billings E, Knox JR, Levesque RC. Site-saturation mutagenesis and three-dimensional modelling of ROB-1 define a substrate binding role of Ser130 in class A beta-lactamases. Protein Eng. 1992 Oct;5(7):693-701.

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Juteau JM, Cote S, Levesque RC. Systematic site-saturation mutagenesis of ROB-1 beta-lactamase: efficiency of T4 polymerase and oligonucleotide synthesis. Biotechniques. 1991 Oct;11(4):460-2.

Juteau JM, Sirois M, Medeiros AA, Levesque RC. Molecular distribution of ROB-1 beta-lactamase in Actinobacillus pleuropneumoniae. Antimicrob Agents Chemother. 1991 Jul;35(7):1397-402.

Juteau JM, Levesque RC.Sequence analysis and evolutionary perspectives of ROB-1 beta-lactamase. Antimicrob Agents Chemother. 1990 Jul;34(7):1354-9.

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Research article

Vaginal microbicides: detecting toxicities *in vivo* that paradoxically increase pathogen transmission

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Abstract

Background: Microbicides must protect against STD pathogens without causing unacceptable toxic effects. Microbicides based on nonoxynol-9 (N9) and other detergents disrupt sperm, HSV and HIV membranes, and these agents are effective contraceptives. But paradoxically N9 fails to protect women against HIV and other STD pathogens, most likely because it causes toxic effects that increase susceptibility. The mouse HSV-2 vaginal transmission model reported here: (a) Directly tests for toxic effects that increase susceptibility to HSV-2, (b) Determines *in vivo* whether a microbicide can protect against HSV-2 transmission *without* causing toxicities that increase susceptibility, and (c) Identifies those toxic effects that best correlate with the increased HSV susceptibility.

Methods: Susceptibility was evaluated in progestin-treated mice by delivering a low-dose viral inoculum (0.1 ID_{50}) at various times after delivering the candidate microbicide to detect whether the candidate *increased* the fraction of mice infected. Ten agents were tested – five detergents: nonionic (N9), cationic (benzalkonium chloride, BZK), anionic (sodium dodecylsulfate, SDS), the pair of detergents in C31G (C_{14}AO and C_{16}B); one surface active agent (chlorhexidine); two non-detergents (BufferGel®, and sulfonated polystyrene, SPS); and HEC placebo gel (hydroxyethylcellulose). Toxic effects were evaluated by histology, uptake of a 'dead cell' dye, colposcopy, enumeration of vaginal macrophages, and measurement of inflammatory cytokines.

Results: A single dose of N9 protected against HSV-2 for a few minutes but then rapidly increased susceptibility, which reached maximum at 12 hours. When applied at the minimal concentration needed for brief partial protection, all five detergents caused a subsequent increase in susceptibility at 12 hours of ~20–30-fold. Surprisingly, colposcopy failed to detect visible signs of the N9 toxic effect that increased susceptibility at 12 hours. Toxic effects that occurred contemporaneously with increased susceptibility were rapid exfoliation and re-growth of epithelial cell layers, entry of macrophages into the vaginal lumen, and release of one or more inflammatory cytokines (Il-1β, KC, MIP 1α, RANTES). The non-detergent microbicides and HEC placebo caused no significant increase in susceptibility or toxic effects.

Conclusion: This mouse HSV-2 model provides a sensitive method to detect microbicide-induced toxicities that increase susceptibility to infection. In this model, there was no concentration at which detergents provided protection without significantly increasing susceptibility.

Background

The healthy, intact vaginal epithelium provides a significant barrier against infection: To transmit infection reliably, 10,000 times more SIV must be delivered to the macaque vagina than that required intravenously [1], and in humans, heterosexual transmission of HIV is estimated to occur in only ~1/1000 coital acts [2]. Even when the male partner is in the highly infectious acute phase, transmission is estimated to occur in only ~1/50 coital acts [3]. Thus any microbicide that has a toxic effect that compromises the vaginal barrier can cause a major increase in susceptibility. To help identify promising microbicide candidates for future efficacy trials, preclinical screening tests are needed that will disclose any toxic effect that increases vaginal susceptibility to infection. At present, screening for toxic effects *in vivo* is most often performed in the primate, rabbit, and mouse vaginal irritation models [4-9]. These models employ histology, colposcopy, and the release of inflammatory cytokines to detect toxic effects [4-6,8-12]. Although of obvious importance, these toxic effects serve only as surrogates for susceptibility, and thus they may fail to disclose toxic effects that significantly increase susceptibility to infection. Fortunately, animal models can be devised to test directly whether a microbicide increases susceptibility.

Some readily observed toxic effects are very likely to increase susceptibility, such as ulceration and inflammatory conditions that increase access to target cells, but there may also be additional susceptibility-increasing toxic effects that have not yet been identified or observed; for example, toxic effects that increase the density of cell-surface receptors on target cells, or that increase the amplification of nascent infections. With this in mind, we developed a mouse vaginal model to detect whether candidate microbicides cause, *by any mechanism*, toxicity that increases the susceptibility of the vagina to HSV infections. HSV-2, like HIV, is an enveloped virus that can transmit infections through the epithelium, and genital herpes is a major STI that is a major co-factor for HIV transmission [13]. HSV-2 is a human pathogen that readily infects many other species, including mice, by binding to highly conserved entry receptors, especially nectin-1, on epithelial target cell surfaces. For example, human, bovine, and porcine herpes viruses can all use human, mouse and porcine forms of nectin-1 for cell entry [14]. Moreover, the course of infection is similar in mice and humans. These characteristics strongly suggest that candidate microbicides that increase HSV susceptibility in mice will likely increase HSV susceptibility in humans.

HIV and HSV both bind initially to heparan sulphate-like molecules on the surfaces of their target cells [15], and although these viruses then use different entry receptors, toxic effects that increase the presence of, or access to, heparan sulphate receptors on their target cells might possibly increase susceptibility to both viruses. Similarly, sulfonated and sulfated polysaccharides (PRO 2000, SPS, cellulose sulphate, carrageenan), bind to both viruses, and PRO 2000 protects against both HIV [16] and HSV [17] in animal models and against both HIV and HSV in human ex vivo tests [18]. Thus toxic effects that increase HSV susceptibility in the mouse may in some cases also reveal toxic effects that increase HIV susceptibility [19].

A key paper by Phillips and Zacharopoulus [20] on susceptibility to rectal transmission of HSV in the mouse revealed that nonoxynol-9 (N9) not only failed to protect, but caused rapid exfoliation of sheets of epithelial cells and greatly increased rectal susceptibility. Phillips and colleagues then found [21,22] that N9 causes similar rapid exfoliation of rectal epithelium in humans. Their results strongly suggest that N9 should not be used for rectal protection in humans. In testing microbicides for protective efficacy in a mouse chlamydia model [23], we found that chlorhexidine (CHX), a widely used microbicidal preservative, causes a 100-fold increase in vaginal susceptibility to Chlamydia trachomatis when tested three *days* after a single vaginal application of this surface-active agent. Thus mouse models for vaginal transmission of HSV and chlamydia provide a way to detect *in vivo* toxicity that increases vaginal susceptibility to viral as well as bacterial STD pathogens. The protective efficacy of many candidate microbicides has been tested for HSV and Chlamydia in a variety of mouse models [7,24-28]. The present investigation is the first designed to: (a) Detect susceptibility-increasing toxicities by observing susceptibility as a function of time following a single application of candidate microbicides, (b) Test whether there is a dose range of a candidate microbicide that can prevent vaginal HSV transmission without causing toxicities that increase susceptibility to HSV, and (c) Monitor observable toxic effects that could potentially be used in clinical (Phase I) evaluations of candidate microbicides to identify toxic effects that best correlate with toxicities that increase vaginal susceptibility to HSV.

Methods

Microbicide candidates

A cross-section of candidate microbicides was selected, some of which have been reported to protect against HSV-2 in mouse vaginal transmission models: N9 [28], SPS [26] (which is similar to T-PSS [24]), chlorhexidine [26], and BufferGel [29]. Others were selected based on published *in vitro* tests: C31G inactivates HSV-2 [30] and both detergents in C31G, ($C_{14}AO$+$C_{16}B$), inactivate HIV [4,31]; BZK and SDS both inactivate HIV [10,31]. Several detergents were selected since detergents, especially N9, are the candidates for which the most clinical data are available. All five of the detergents are comparably potent at inacti-

vating HSV, HIV, or sperm *in vitro*: ~0.01–0.03% rapidly disrupts envelope and cell membranes [4,32-34]. Nonoxynol-9 is a non-ionic detergent used in most currently available vaginal spermicides and detergent-coated condoms, and is widely used in disinfectant handwashes. Benzalkonium Chloride (BZK) is a cationic detergent with exceptionally broad microbicidal action and is a component of the "Protectaid®" contraceptive sponge. Sodium Dodecyl Sulfate (SDS, also called sodium lauryl sulfate, SLS) is an anionic detergent widely used in toothpastes, shampoos and shaving creams. C31G is an equimolar mixture of two detergents (C_{14}AO, C14 alkyl amine oxide and C_{16}B, C16 alkyl betaine) used as an oral disinfectant in dentistry and as the active ingredient in "Savvy", a spermicidal microbicide now in clinical efficacy trials for contraception and HIV prevention. Chlorhexidine (CHX) is a surface active agent that is even more potent than the above detergents and is used as a preservative in K-Y Jelly® and Surgilube®, and as a disinfectant in mouthwashes and antimicrobial handwashes. BufferGel® is a spermicidal microbicidal gel made with Carbopol 974P, a cross-linked polyacrylic acid polymer, and formulated at pH 3.9 to match the pH of the healthy human vagina. It has adequate buffer capacity to acidify the ejaculate and thereby reinforces the protective acidity of the vagina. Sulfonated polystyrene (SPS) is a polymer similar to T-PSS [24], and has potent spermicidal and antiviral efficacy in vitro [26]. It is similar, to some extent, to the sulfated and sulfonated polysaccharides now in clinical HIV prevention trials (Carraguard, PRO 2000, and cellulose sulphate). These candidates were obtained from: N9 (Rhone-Poulenc, Cranbury NJ); BZK and chlorhexidine (Sigma, St. Louis, MO); SDS (Invitrogen Corp., Carlsbad, CA); C_{14}AO (myristyl dimethylamine oxide, Chemron, Pasa Robles, CA.) and C_{16}B (cetyldimethylbetaine, DeForest Enterprises, Inc., Boca Raton, FL); BufferGel® and HEC placebo gel [35] (ReProtect, Inc., Baltimore MD); SPS (polystyrene sulfonate, average molecular weight 500,000: Scientific Polymer Products, Inc., Ontario, NY). With the exception of BufferGel and the HEC placebo gel, all agents were tested for toxicity as 2% solutions in phosphate buffered saline (PBS) to avoid the confounding effects of differing formulations. Similarly, for determining the acute toxicity and protective efficacy, the detergents and CHX were also delivered in PBS but at various concentrations as needed.

Mouse model

Progestin treatment

Female CF-1 mice 6–8 weeks old (Harlan, Indianapolis, IN) were acclimatized for 1–2 weeks after shipping, then injected subcutaneously with 2.5 mg Depo-Provera® (medroxyprogesterone acetate) (Pharmacia & Upjohn Company, Kalamazoo, MI.), a treatment that produces a diestrous-like state that eliminates the stratified squamous layer of dead and dying cells that otherwise helps protect the vagina. In this diestrous-like state the epithelium becomes similar to columnar epithelium in that the entire epithelial surface becomes covered with living cells. This progestin treatment greatly increases HSV susceptibility and makes mice more uniform in susceptibility than randomly cycling mice [26,28,36]. Depo-Provera makes the mouse vagina more closely mimic the most accessible HSV target cells in the human female genital tract, the columnar epithelial cells of the endocervical canal and regions of cervical ectopy that occur commonly in younger women, regions in which living cells are exposed directly on the face of the cervix.

Viral inoculum

Strain G of HSV-2 (ATCC lot #3405329) was obtained from Virotech International (Rockville, MD; 5×10^8 $TCID_{50}$/ml). The viral stock was thawed and refrozen in 100 µl aliquots, then stored at -70°C. A thawed aliquot of viral stock was diluted with Bartels Tissue Culture Refeeding Medium (Trinity Biotech, St. Louis, MO) to yield an inoculum with 10 ID_{50} in a 10 µl inoculum (~10^4 $TCID_{50}$). For low-dose inocula, the viral stock was further diluted with Bartels Medium as needed. The diluted viral stock was stored on ice and used within 1 hour of thawing. The 10 µl viral inoculum was delivered with a Wiretrol pipet (Drummond Scientific, Broomall, PA) with a fire-polished tip to minimize potential injury.

Assay for infection

As reported earlier [26,28,29], vaginal lavages were obtained 3 days after inoculation and evaluated for viral shedding. Input virus from the inoculum can not be detected for more than 6 hours after the inoculation, and viral shedding by infected animals reaches a maximum at 3 days (data not shown). This assay is more rapid and also more sensitive than waiting for visible lesions or death [26], both of which are more dependent on the hormonal and immune status of the mouse [37]. Importantly, assay of infection at day 3 avoids causing animal pain. Fifty µl of Bartels Medium was delivered to the vagina and pipetted in and out 20 times to maximize viral recovery, then diluted into 50 µl Bartels Medium in a 0.5 ml microfuge tube. The vaginal lavage samples were then spun at 6500 rpm for 5 minutes to pellet the cells and mucus. The pellet was then removed using a pipet tip to draw the pellet up the side of the tube and out of the supernatant. The supernatant was then placed on target cells (human newborn foreskin diploid fibroblast cells; Biowhitaker, Walkersville, MD). Cytopathic effect was scored 48 hours later, and mice whose lavage cultures displayed cytopathic effect were considered infected. The entire test thus requires a total of 5 days from inoculation to assay of infection, making this a relatively rapid and efficient animal model.

Protection assay

Two procedures were used to observe the protective efficacy of the test agents. In the "mix externally" procedure, the 10 μl viral inoculum with 10 ID_{50} was mixed together with 20 μl of the test agent, incubated for 5 minutes at 37°C, and then this mixture was delivered to the vagina using a 50 μl Wiretrol pipet with fire polished tip. This method insures the virus is fully exposed to the test agent before it can contact target cells. In the "mix in vagina" procedure, which is intended to mimic human use of a microbicide, 20 μl of the test agent (or PBS) was delivered to the vagina and the 10 μl viral inoculum with 10 ID_{50} was delivered after a specified time interval. In most experiments 30 mice were divided into groups of 10, one group treated with one test agent, a second group with a second test agent, and the third (control) group was treated with PBS (phosphate buffered saline). Each such experiment was repeated two or three times to obtain results from 20 to 30 mice for each test agent.

Susceptibility assay

In this procedure, 20 μl of the test agent was delivered to the vagina, and then a low-dose inoculum with 0.1 ID_{50} was delivered in 10 μl of Bartels medium after a specified time interval. To determine the relative susceptibility of the mice, two groups of control mice (treated with PBS at the same time interval) were used, one group inoculated with the same 0.1 ID_{50} low-dose inoculum and one inoculated with 10 ID_{50}. On average, the low-dose inoculum infected 13.5% of the control mice; the high-dose inoculum infected 87%. For each test agent, the fraction infected in each of its control groups was used to construct a dose-response graph (fraction infected vs log ID) and drawing a linear interpolation between the fractions infected by the 0.1 and 10 ID_{50} inoculations. The fraction of mice infected in the test group was then plotted on this graph to determine the effective ID of the low-dose inoculum. Relative susceptibility is defined as the ratio of the effective ID the low-dose inoculum delivered to the test mice divided by the ID it delivered to control animals. For example, if the low-dose 0.1 ID_{50} inoculum infected 50% of the test mice, it acted effectively as 1 ID_{50} indicating the relative susceptibility of the test mice increased 10 fold. In most experiments 30 mice were divided into 3 groups of 8 (two test groups and 1 control group) all of which received the low-dose 0.1 ID_{50} inoculum, and one control group of 6 received the high-dose10 ID_{50} inoculum. Each experiment was repeated to obtain ~30–50 mice per group to insure robust statistical power.

Acute toxicity

Dead cell dye

To detect acute toxicity to the epithelial cells, the vagina was stained with YOYO®-1 (Molecular Probes, Inc., Eugene, OR) a membrane impermeant dye that can enter the nuclei, and thereby become highly fluorescent, in cells whose membranes are disrupted. To keep the mice immobile throughout this test procedure, they were first anesthetized by intraperitoneal injection of 0.5 ml "Avertin" (2.5 g 2,2,2 tribromoethanol in 5 ml amylene hydrate and 200 ml water). Fifty μl of the test agent (or PBS control) was delivered with a Wiretrol pipet with fire-polished tip, and the agent left in place for 10 minutes. The vagina was then gently lavaged for 10 minutes using a syringe pump to continuously deliver PBS through a fire-polished Wiretrol pipet at a rate of 5 ml/minute. Then 20 μl of YOYO-1 (diluted to 5 μM with PBS) was delivered and left in place for 10 minutes before again lavaging the vagina for 10 minutes with PBS to remove unbound dye. The mouse was then sacrificed, the vagina dissected out and slit open lengthwise, and again rinsed for 10 minutes in PBS. The entire vagina was then mounted between two microscope slides that were squeezed together to flatten the vagina and open its numerous folds (rugae). Digital images of the flat-mounted vagina were taken with a macro-lens (1×) in a Nikon E800 epifluorescence microscope.

Histology

Groups of 4 mice were treated intra-vaginally with 20 μl 2% N-9 (or PBS for controls) and sacrificed at various times immediately before dissecting out the vagina. One N9 group and one control group were dissected at 10 minutes, and additional N9 groups were dissected 2, 8, 12, 18, and 24 hours post-exposure. Each vagina was fixed in 5 ml 10% neutral-buffered formalin (Sigma-Aldrich, St. Louis, MO). The vaginas were embedded, sectioned transversely at four regions, stained with hemotoxylin and eosin, examined by bright-field microscopy, and evaluated for average number of cell layers in the epithelium, and scored semi-quantitatively for quantity of visible cellular material in the lumen.

Cytokine assay

The Bio-Plex system (Bio-Rad, Hercules, CA) was used to measure mouse inflammatory cytokine concentrations in vaginal lavage samples. Inflammatory cytokines included in this study were those shown to be informative in human vaginal microbicide studies [38] and for which there were mouse cytokine reagents available for the Bio-Plex assay. Those tested were: IL-1α, IL-1β, Il-6, KC (murine equivalent of IL-8), MIP-1α, and RANTES. The vagina was lavaged repeatedly (pipetted in and out 20 times) with 50 μl PBS. The lavage fluid was then centrifuged, as described above, to obtain the supernatant which was frozen immediately and stored at -70°C until analyzed. Samples were diluted 1:3 with PBS prior to assay. Cytokine concentrations were determined using the calibration procedure and cytokine reference standards supplied with the Bio-Plex assay. Interference control tests were performed by spiking ten-fold serial dilutions of 2%

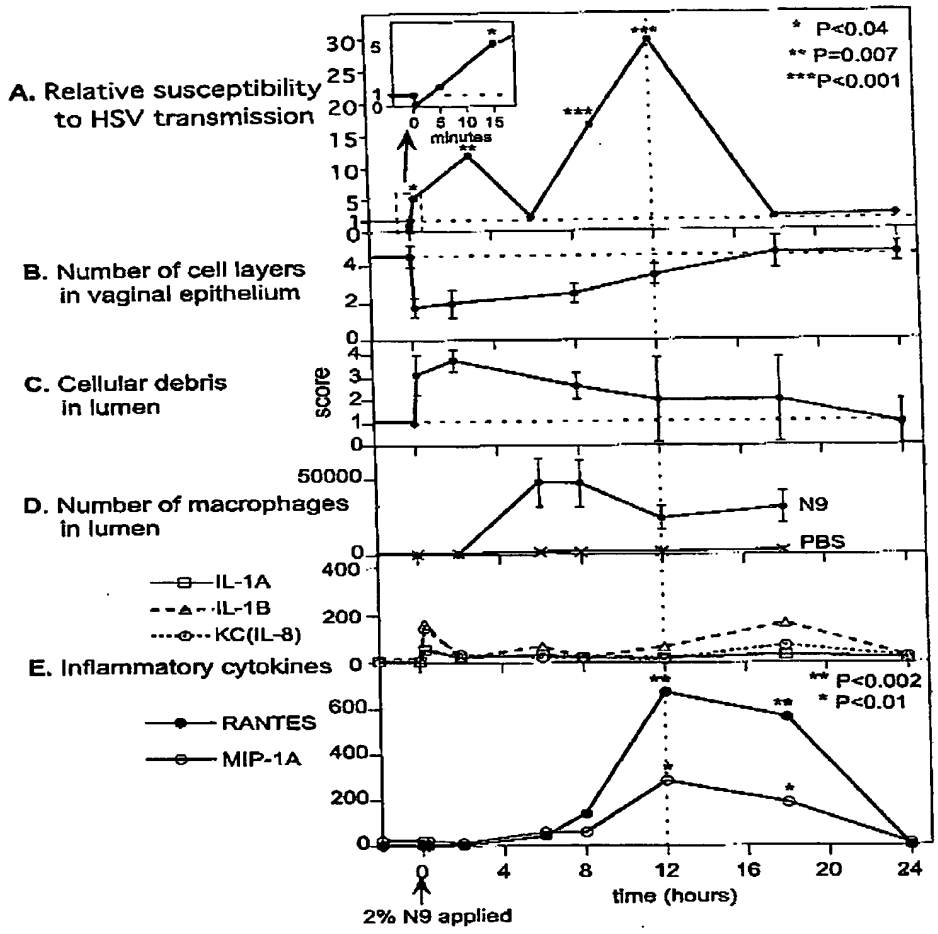

Figure 1
Vaginal HSV-2 susceptibility and toxic effects vs time after a single dose of 2% N9. A. Relative susceptibility is the effective Infectious Doses the inoculum delivers in N9-exposed mice normalized to the Infectious Doses it delivers in control (PBS) mice. The single dose of N9 produced three successive phases in susceptibility, a brief initial protective phase (reduced susceptibility) followed by two distinct phases of markedly increased susceptibility. *P* values were determined by Fisher's exact two-sided test comparing the numbers of animals infected and uninfected in the test animals vs. the corresponding numbers in control animals treated only with PBS. **B-E.** Error bars indicate standard deviations around the mean. Concentrations of inflammatory cytokines are indicated by geometric means normalized with respect to those for PBS controls. See legend for Fig. 6 for geometric means of PBS controls. **B, C.** Data obtained from histology sections from four locations distributed along the length of the vagina. The score in **C** is a semi-quantitative evaluation of the amount of visible cellular debris in the vaginal lumen. For each point in **B** and **C** at 10 min, 2, and 8 hours, *P* values were <0.001 with respect to PBS control values. (n = 8) **D.** *P* values for all times points from 6 to 18 hours were <0.001 with respect to PBS control values. (n = 20) **D, E.** Data obtained from vaginal lavage samples.

microbicide samples with defined amounts of cytokine standards. SPS was the only microbicide that inhibited cytokine detection within the concentration range expected to be found in vaginal samples; detection of KC, MIP-1alpha and RANTES was inhibited >50% in the presence of SPS at concentrations ranging from 0.2% to 0.002%.

Colposcopy

Mice were anesthetized with Avertin (as described above), immobilized with tape on a custom-made platform, and the vagina was held open by means of two small spring-loaded forceps acting like a speculum with four blades. A colposcope (Zeiss OPMI 1-SH) was set at maximum magnification (31×) and focused on the epithelium surrounding and including the cervix. Direct visual observations were made by four observers who were not informed of the agent to which each mouse had been exposed. In addition, photographic images were obtained, and digitized for later analysis. All observations were made with carefully controlled standard illumination, and all images were processed and displayed by identical procedures; no adjustments of color balance or contrast were made to the digitized images.

Statistics

GraphPad Instat version 3 (San Diego, CA) was used for statistical analysis. Fisher's exact two-sided test was used to analyze numbers of mice infected and uninfected in test groups vs control groups in the protective efficacy and susceptibility experiments. The changes at each time point in the number of cell layers, cellular debris in the lumen, and number of macrophages in the lumen, were compared with those for PBS controls using the unpaired t-test for 2-tailed P value. For cytokines, concentrations in the lavage samples were converted to $\log_{10}$ concentrations, and compared with those for PBS controls using the unpaired t-test for 2-tailed P value.

Results

Time course of HSV susceptibility after a single application of N9

Panel A in Fig. 1 shows susceptibility to HSV as a function of time after delivering a single dose of 2% N9. The small inset shows that immediately after delivery N9 reduced susceptibility about 6-fold (partial protection), but by 5 minutes the mice were no longer protected since the fraction of mice treated with N9 that became infected was larger than the fraction of the control mice treated with PBS. At 15 minutes mice treated with N9 became about 5 times more susceptible than the controls ($p = 0.04$). Susceptibility rose to a peak at about 3 hours, then declined to the baseline susceptibility of PBS controls at 6 hours. Susceptibility then rose again reaching a major peak at about 12 hours before declining to baseline at 18 and 24 hours. The decrease in susceptibility at 6 hours suggests there are two separate phases of increased susceptibility. Note that at the 12 hour peak, a single dose of N9 increased susceptibility nearly 30-fold ($P < 0.001$), that is, to infect 50% of the N9 exposed mice, a viral inoculum that infects 50% of the control mice would have to be reduced in viral titer by 30-fold.

Time courses of toxic effects that might correlate with increases in susceptibility

N9 caused rapid exfoliation of epithelial cells, as indicated in Fig 1B by the reduction in number of epithelial cell layers from 4–5 to 1–2 (a loss of about 3 cell layers) and in Fig 1C by the rapid increase in cellular debris in the vaginal lumen. The cell layers slowly regenerated and returned essentially to that of the controls by 18 hours. Note that the initial rapid rise in susceptibility that started at ~5 minutes and peaked at ~3 hours was contemporaneous with the exposure of deeper cell layers in the vagina, and that the subsequent major increase in susceptibility that started after 6 hours, peaked at 12 hours, and ended prior to 18 hours was contemporaneous with the duration in which the layers of cells were regenerating.

N9 also caused a delayed but large influx of macrophages into the vaginal lumen as detected in vaginal lavage samples, Fig 1D. The peak macrophage content of the lumen occurred at about 6–8 hours and somewhat preceded the major peak in HSV susceptibility. Note also that during the initial rapid rise in susceptibility no macrophages were detected in the vaginal lumen, and also that macrophages persisted in the lumen at a greatly elevated level at 18 hours, when the susceptibility had returned to that of the controls.

Fig. 1E shows the time course of inflammatory cytokines recovered in vaginal lavage fluid at the times indicated (each mouse was lavaged once). No Il-6 was detected at any time, and Il-1α did not change detectably at any time. There were non-significant trends towards an immediate increase of IL-1β and KC (murine equivalent to IL-8) followed much later at 18 hours by another non-significant trend toward an increase in IL-1β. In contrast, RANTES and MIP-1α both exhibited a delayed and major increase starting at about 8 hours, peaking at about 12 hours, and persisting at significantly increased levels at 18 hours (a time when the susceptibility had returned to normal).

Vaginal HSV susceptibility 12 hours after a single application of candidate microbicides

Since the major peak in susceptibility caused by toxic effects of N9 occurred about 12 hours after delivering this detergent, the other microbicide candidates were also tested 12 hours after applying a single dose. The two detergents in C31G, $C_{14}AO$ and $C_{16}B$, were tested separately

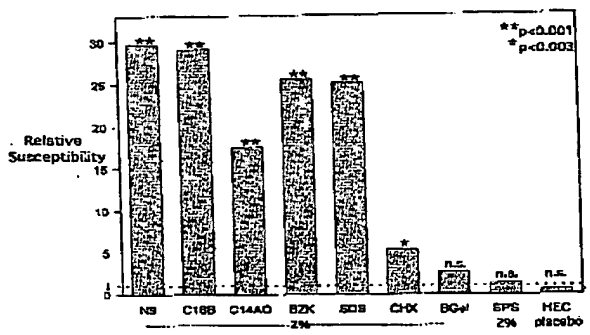

Figure 2
Vaginal HSV-2 susceptibility 12 hours after a single application of candidate microbicides. *P* values (from Table 1), determined by Fisher's exact two-sided test as described in the caption for Figure 1. n.s. = not significant.

since they have been reported to differ significantly in their toxic effects [4]. The results are summarized in Fig. 2. All five detergents (N9, $C_{14}AO$, $C_{16}B$, BZK, and SDS) caused comparable major increases in susceptibility (18–29 fold; $P < 0.001$). The susceptibility increases caused by $C_{14}AO$ and $C_{16}B$ did not differ significantly even though $C_{16}B$ has been reported to produce a less intense inflammatory response than $C_{14}A$ [4]. The surface-active agent, CHX, caused a smaller but significant 5-fold increase ($P < 0.003$). In contrast, the small susceptibility changes following exposure to BufferGel, SPS, and the HEC placebo were not significant. The data on which Fig 2 is based are listed in Table 1.

A single prior exposure to N9 can abolish its transient protective effect

The disappointing results of the clinical trials of N9 products for HIV prevention led to the suggestion that persistent toxic effects might increase susceptibility during coital events when the product was not used. If this is the major problem caused by N9 toxicity, then "user failure" is of crucial importance. However, the present results suggest an additional possibility, namely that detergent toxicity may cause such rapid and large increases of susceptibility that even when used correctly they may fail to protect. Most N9 products are recommended to be used within 1 hour of application, but as shown in Fig. 1A, N9 significantly increased susceptibility within 15 minutes of application. A second test of the hypothesis that detergents may not protect even when used correctly is shown in Fig. 3.

Mice were exposed to a single dose of 2% N9, or PBS, and then 12 hours later were treated with N9, or PBS, just before delivering a *low-dose* viral inoculum (0.05 ID_{50}). As can be seen in Fig. 3, the low-dose inoculum infected 4 of 32 control mice (exposed twice to PBS), and 1 of 32 positive control mice (PBS followed 12 hours later by 2% N9 delivered just prior to the inoculum). In contrast, 9 of 32 test mice previously exposed to N9 became infected, even though in this test group a second dose of N9 was delivered just prior to the low-dose inoculum to mimic correct use of a microbicide: (9 of 32 vs 1 of 32; P = 0.013). Thus a single earlier dose of N9 abolished the transient protective effect of the subsequent dose of N9 even when used correctly.

Acute toxic effects of detergents

Detergents can rapidly disrupt the membranes of living cells exposed on the epithelial surface, and Fig. 4 shows the effects of a 10-minute exposure to 2% BZK, 2% N9, and PBS applied to the vagina of progestin-treated mice. The entire vaginas, opened and flat-mounted, were stained with the "dead cell" dye YOYO-1 (Molecular Probes, Eugene, OR) that makes nuclei of cells with disrupted membranes intensely fluorescent. Both detergents caused large swaths of the epithelial surface to become intensely fluorescent, regions in which virtually every cell nucleus became highly fluorescent. In contrast, PBS caused little detectable damage.

The images graphically reveal the extent of acute damage caused by these detergents to the living cells on the epithelial surface, unprotected by layers of senescent squamous cells. Note that the swaths of fluorescent cell nuclei visible in this figure were in cells located in *deeper* layers of the epithelium since the detergents caused the outermost cell layers to be rapidly shed, as shown above in Fig. 1B and 1C. Also noteworthy is that the detergents failed to enter several regions of the vaginal surface, as indicated by the dark, unstained bands. These bands were located on the interior surfaces of the vaginal rugae (folds); these bands became fully visible only when the vagina was flattened (or stretched laterally) sufficiently to spread out the rugae. Thus the 10-minute exposure to the detergents was not sufficient for detergents to enter into the rugae of the vagina. This result was similar to an earlier study using ethidium homodimer-1 instead of YOYO-1 [23]. In subsequent studies (data not shown), we found that even if a large ball is placed on the tip of the pipet to help spread out the rugae, and the pipet is stirred with a variety of motions, the detergents still fail to enter many of the rugae. This incomplete "deployment" of vaginal microbicides into the many folds of the vaginal epithelium probably contributes to the inability to achieve complete protection in tests of candidate microbicides in animal models [23]. Moreover, as shown in the following section, incomplete deployment probably contributes to incomplete protection even when microbicide agents are deliv-

Table 1:

agent or PBS	agent then 0.1 ID_{50}		PBS then 0.1 ID_{50}		PBS then 10 ID_{50}		Relative Suscept.	P
12 hours earlier	infected	inoculated	infected	inoculated	infected	inoculated		
2% N9	29	42	8	40	35	40	29	<0.001
2% $C_{16}B$	20	30	4	30	25	29	29.1	<0.001
2% $C_{16}AO$	25	41	4	39	34	37	17.5	<0.001
2% BZK	28	40	9	41	37	41	25.5	<0.001
2% SDS	25	37	4	36	34	37	25.0	<0.001
2% CHX	15	45	3	45	35	44	5.4	<0.003
BufferGel	14	52	6	46	39	51	2.7	0.13
2% SPS	5	39	3	34	31	35	1.3	0.7
HEC Placebo	3	32	6	30	30	30	0.5	0.3
2% N9 at time earlier								
5 minutes	8	32	5	32	18	18	1.7	0.5
15 minutes	12	28	4	27	26	28	5.2	0.037
3 hours	26	39	14	41	23	27	12	0.007
6 hours	15	45	10	43	31	39	2.3	0.35
9 hours	22	38	3	37	35	39	16.6	<0.001
18 hours	9	37	5	34	31	36	2.2	0.4
24 hours	8	29	4	29	24	28	2.4	0.3
				2% N9 then 10 ID_{50}				
30 seconds	10	18			17	20	0.16	0.07

ered at concentrations much higher than needed to inactivate pathogens in vitro.

Selectivity index in vivo

Animal models provide the best available method for predicting whether a candidate microbicide will be sufficiently selective to merit tests in human clinical trials. An *in vivo* selectivity index can indicate whether or not a test agent can provide protection without causing unacceptable toxic effects.

Fig. 5 illustrates methods for determining a selectivity index. The upper panel shows a measure of acute toxicity, the average fluorescence intensity of the entire vagina stained with YOYO-1 as above (Fig. 4) as a function of the concentration of the detergent to which the vaginas had been exposed for 10 minutes. BZK and N9 caused detectable increases in fluorescence starting at about 0.05% and markedly increased fluorescence at 0.2% and above, indicating major regions of epithelial cells had disrupted membranes. (Other detergents we tested, especially SDS, an anionic detergent, significantly blocked fluorescence by YOYO-1, a cationic dye, and hence acute toxicity caused by these detergents could not be evaluated with this method. Apparently, despite the extensive and repeated washing procedure, significant amounts of detergents must have been retained by epithelial cells, perhaps by being intercalated into cell membranes at concentrations too low to solubilize the membranes but high enough to interfere with fluorescence of YOYO-1.)

The lower panel in Fig. 5 shows two methods for testing the protective efficacy of a candidate microbicide in the mouse HSV model. The "mix externally" method is similar to cell-based *in vitro* tests; the viral inoculum is thoroughly mixed together with the test agent *before* the mixture is delivered to the vagina. This insures that the viral inoculum is well exposed to the test agent before the virions can contact target cells. The "mix in vagina" method is designed to mimic human use of microbicides; the candidate microbicide is delivered to the vagina first, and then the viral inoculum is delivered.

As expected on the basis of cell-based tests, the detergents were significantly protective in the "mix externally" method at the minimal concentrations that inactivate HSV and HIV virions *in vitro*, ~0.03% [31,33]. At a slightly higher concentration, ~0.05%, the detergents disrupted cell membranes in the epithelium, as indicated by increased YOYO-1 fluorescence of the vagina. In marked contrast, but also as expected from previous tests in animal models [26,39,40], in the "mix in vagina" method the

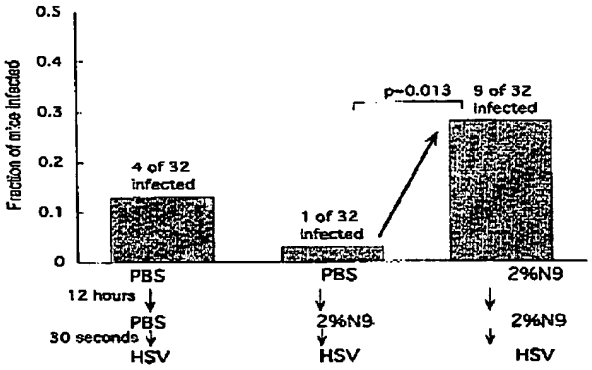

Figure 3
A single previous exposure to N9 can abolish the brief protective effect of N9. The low-dose inoculum (0.05 ID_{50}) infected only a small fraction of the control group treated twice with PBS (first column), and an even smaller fraction was infected in the control group treated with 2% N9 just before delivering the inoculum (middle column). But a significantly larger fraction became infected in the test group (right-hand column), revealing that the N9 dose delivered 12-hour earlier abolished the protective effect of the N9 dose delivered just before the inoculum: P = 0.013 by Fisher's exact two-sided test.

detergents failed to protect unless delivered at concentrations ~1000-fold higher (~3%). By comparing the upper and lower panels in Fig. 5 it can be seen that detergents became acutely toxic at much lower concentrations than the concentrations needed for significant protection. Most important, the detergents provided only partial, if any, protection at 2%, a concentration at which all the detergents markedly increased HSV susceptibility as shown above in Fig. 2. Thus in this mouse HSV model there was no concentration at which N9, BZK, $C_{14}AO$, $C_{16}B$, and SDS were protective without causing unacceptable toxicity.

Inflammatory cytokines

Prior to Phase II/III efficacy trials, there is a critical need for tests that can be performed in Phase I safety trials to screen out candidates likely to cause unacceptable toxicity. One such screening test is to observe the release of inflammatory cytokines since inflammatory responses are likely to increase susceptibility to HIV and other pathogens [10,38]. In the mouse HSV model, toxic effects that increase susceptibility can be directly measured and correlated with releases of inflammatory cytokine responses to help determine which increases in cytokines correlate best with increases in susceptibility to HSV infections. Therefore, in experiments performed in parallel with those shown in Fig. 2, vaginal lavage samples were obtained 12 hours after a single exposure to the candidate microbicides and analyzed for inflammatory cytokines. The results are summarized in Fig 6. The detergents were all tested at a total concentration of 2%, since this was the minimal concentration at which these detergents provided partial transient protection. Twelve hours after a single application, all detergents tested produced a significant increase in one or more inflammatory cytokines: All significantly increased RANTES; N9, ($C_{14}AO$+$C_{16}B$), and SDS significantly increased MIP-1α. In addition, ($C_{14}AO$+$C_{16}B$) significantly increased IL-1β, and SDS significantly increased KC (murine equivalent of Il-8). Thus all detergents, as tested here, caused significant increases both in HSV susceptibility and release of one or more inflammatory cytokines. Chlorhexidine (CHX) caused a less marked increase in HSV susceptibility and only statistically insignificant trends in Il-1β, KC, and MIP-1α. In contrast to the detergents, BufferGel, SPS, and the HEC placebo did not significantly increase susceptibility nor cause a significant release of inflammatory cytokines. SPS also did not increase susceptibility, but cytokine proinflammatory results are inconclusive because unlike the other agents, SPS significantly interfered with the detection of several of the cytokines in the Bioplex assay.

In summary, by observing a broad range of inflammatory cytokines there emerged a significant, but non-quantitative correlation between susceptibility-increasing toxicity and the elevation of inflammatory cytokines. But note that the time-courses were *not* well correlated.

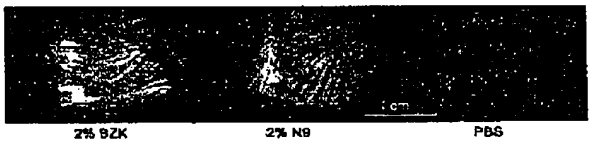

Figure 4
Surface distribution of acute toxicity to vaginal epithelium caused by a 10-minute exposure to detergents. Fluorescent intensity of the 'dead cell' dye YOYO-1 is proportional to the density of cell nuclei in the epithelium whose membranes were disrupted by the detergents. The brightly stained swaths of epithelium are regions in which virtually every cell nuclei became stained with YOYO-1. The darker regions became visible as the vagina was gently stretched and flattened to unfold the rugae.

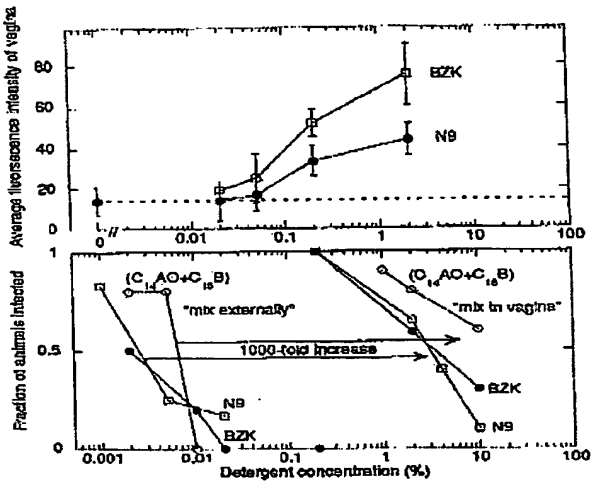

Figure 5

Acute toxicity, and fraction of mice infected, vs detergent concentration. The upper panel shows the average fluorescent intensity of the YOYO-1 stained vagina as a function of the concentration of detergent to which each vagina was exposed for 10 minutes. The lower panel shows the fraction of mice infected using two different test methods: 'mix externally' fully exposes HSV in a high-dose inoculum (10 ID_{50}) to the microbicide prior to inoculation, and 'mix in vagina' mimics human use of a microbicide, the microbicide is delivered to the vagina before delivering the high-dose (10 ID_{50}) inoculum. The two detergents in C31G, ($C_{14}AO+C_{16}B$), were combined at equal w/v concentrations and plotted at the total detergent concentrations shown.

Colposcopy fails to detect N9 toxicity that increases susceptibility

Any candidate microbicide that causes colposcopically detectable vaginal toxicity is likely to be screened out prior to Phase II/III efficacy trials. But it is not known whether colposcopy can detect all toxic effects that greatly increase susceptibility to HSV or HIV. Fig. 7 shows high-magnification colposcopic images obtained from 10 mice treated either with a single application of 2% N9 or PBS as a control. These images were obtained 12 hours after delivering the detergent (or PBS), the time at which the susceptibility-increasing toxicity of N9 reaches its peak. Four observers independently attempted to use colposcopy to distinguish mice treated with N9 from those treated with PBS. Attention was focused on detecting erythema, ulcerations, and widened capillaries with more diffuse borders. Neither by direct visual examination with the colposcope, nor by careful examination of digitized, greatly enlarged photographic images, could the observers do better than chance in distinguishing the N9-exposed mice from the control mice.

Discussion

Effects of N9 in mice and humans

N9 was thoroughly studied here since it is the microbicidal detergent for which there is the most clinical evidence regarding both its contraceptive efficacy against sperm [41] and also its failure to provide protection against STD pathogens some of which, like HIV, are potently and rapidly inactivated by this detergent [42,43]. N9 is highly potent *in vitro* against both sperm [34] and HIV [33](~0.02% inactivates both in seconds), and N9 is an effective contraceptive. This has led to the conjecture that despite its toxic effects N9 might protect against HIV *if used correctly every time*. However, the results in this mouse model indicate that even with *correct* use, N9 may fail to protect against vaginal HSV infections. For most N9 products it is recommend that coitus take place within 1 hour of application, but as shown in Fig. 1, the duration of protection in the mouse lasted only ~5 minutes, and susceptibility increased significantly by 15 minutes, even though most of the detergent was still present (see [44]). This suggests that in human use susceptibility might actually increase soon after the first application of the detergent. Moreover, with repeated use, the prolonged susceptibility-increasing toxic effects of N9 might abolish even the brief duration of protection it might otherwise provide, as shown in Fig 3.

Taken together with the rectal studies by Phillips and colleagues [20] the increases in vaginal susceptibility caused by all five detergents and by CHX cast doubt on the suitability of detergents and surface-active agents for vaginal as well as rectal protection against HSV. Single applications of N9 and K-Y® jelly to the mouse uterus cause long-lasting toxic effects (exfoliation and regeneration still evident after 48 hours) [9]. CHX, the preservative in K-Y® jelly, not only increases vaginal susceptibility to HSV but also causes a major, and long-lasting (3 days) increase in susceptibility to chlamydia [23]. Since vaginal applications of the detergents cause significant release of inflammatory cytokines in mice, rabbits [10] and women [38], these detergents may also increase susceptibility to HIV infections (see [10,38]). In contrast, both non-detergent candidates tested here, SPS and BufferGel, when applied at a protective dose [26,29], did not cause significant toxic effects.

Epithelial exfoliation and regeneration correlates best with HSV susceptibility

We attempted to identify observable toxic effects that could be used in preclinical and clinical Phase I safety tri-

als to screen out candidate microbicides that cause susceptibility-increasing toxic effects. These included observations of epithelial exfoliation (desquamation) and regeneration, vaginal entry by macrophages, release of inflammatory cytokines, and colposcopy. The toxic effect that best correlated with the time-course and duration of increased HSV susceptibility was the time course of the changes in thickness of the epithelium. The initial rapid increase in susceptibility occurred during the initial rapid exfoliation of epithelial cells (thereby exposing cells deeper in the epithelium). This result is similar to the rapid exfoliation caused by N9 applied to the columnar epithelium of the mouse uterus [9] and mouse rectum [20]. N9 rapidly increases rectal susceptibility in the mouse. Based on the dose-response results reported by Phillips and Zacharopoulos [20], the lowest dose viral inoculum they used, 5×10^3 PFU, delivered ~0.05 ID_{50} to the controls, but acted effectively as ~5 ID_{50} when delivered 5 minutes after applying 2 % N9 to the rectum. Thus 2% N9 increased rectal HSV susceptibility by ~100 fold within 5 minutes of application. The columnar epithelium of the mouse rectum appears to regenerate within ~1 hour [20]. In contrast, the columnar epithelium of the mouse uterus regenerates in ~72 hours [9]. Since the vaginal epithelium became maximally susceptible at ~12 hours, when its cell layers were regenerating, our results suggest that rectal and uterine susceptibility might become maximal during the markedly different rates at which their epithelial cell layers regenerate.

Nectin-1

A possible mechanism for both the early and late phases of increased susceptibility that occur following epithelial exfoliation caused by detergents is suggested by the following findings regarding nectin-1, a major cell-surface entry receptor for HSV: 1) Mice are susceptible to HSV only during the stages of the estrous cycle when the vaginal epithelium expresses nectin-1, and the most susceptible stage can be maintained by treatment with Depo-Provera [45], 2) Incubating HSV with nectin-1 prior to vaginal inoculation blocked infection [45], 3) In the intact epithelium nectin-1 is primarily localized in the lateral surfaces between adjacent epithelial cells, a location where it is relatively inaccessible to HSV. But upon epithelial disruption by low calcium, nectin-1 migrates from the lateral surfaces and becomes accessible to HSV on the apical surface, increasing viral entry by HSV [46]. In the human vagina, nectin-1 is expressed most intensely in deeper cell layers, the stratum spinosum, the layer of maturing cells just above the basal cells, and is expressed closer to the luminal surface during epithelial regeneration in the secretory phase of the cycle [45]. This suggests that rapid desquamation will rapidly increase access of HSV to cells whose surfaces have more nectin-1, and also that during regeneration there will be more nectin-1 on the epithelial surface. Nectin-1 is also heavily expressed on cell surfaces during epithelial remodelling in the mouse embryo [46], which again suggests nectin-1 may be heavily expressed during the regeneration of vaginal epithelial cell layers.

Thus nectin-1 provides a possible mechanism to explain both the rapid initial increase in susceptibility and for the later major increase in susceptibility (compare the time-course for susceptibility, Fig. 1A, with the number of cell layers, Fig 1B.). The rapid initial increase in susceptibility was contemporaneous with the exposure of deeper layers in the epithelium, and the later prolonged increase in susceptibility occurred contemporaneously with epithelial regeneration.

Taken together, these results suggest that women may become more susceptible to HSV within minutes of applying a microbicide that causes rapid exfoliation of columnar epithelium and may become even more susceptible throughout the duration of epithelial regeneration. Human columnar epithelial cells are target cells for HSV, and in the absence of trauma or open sores or ulcers, the most susceptible sites are likely to be the columnar epithelial cells in regions of cervical ectopy and in the endocervix. Uterine peristalsis transports vaginal fluids, including sperm [see [47]] and microbicide gels [48-51], to the columnar epithelium of upper tract. Thus microbicides (and pathogens) both contact columnar epithelium of the upper tract. Even though the vaginal epithelium is protected by multiple layers of dead and dying squamous cells, toxic effects of microbicides applied to columnar epithelium should be carefully considered in the design of Phase I trials.

Which toxic effects related to HSV susceptibility might usefully be monitored in Phase I trials of candidate microbicides?

Exfoliation

Monitoring changes in the thickness of the columnar epithelium in women would require biopsy specimens of ecto- or endocervical tissue taken shortly before and after exposure to the test agent. However, optical coherence tomography (OCT, [52]) might provide a non-invasive method for detecting ecto-cervical exfoliation, and it might also be possible to detect rapid epithelial exfoliation by collecting lavage or swab samples directly from the ectocervix and cervical os before and shortly after applying the candidate microbicide and looking microscopically for epithelial sheets.

Leukocyte entry

Fichorova, Tucker, and Anderson [38] reported that N9 caused macrophages and neutrophils to enter the human vaginal lumen in large numbers. Milligan et al. [8]

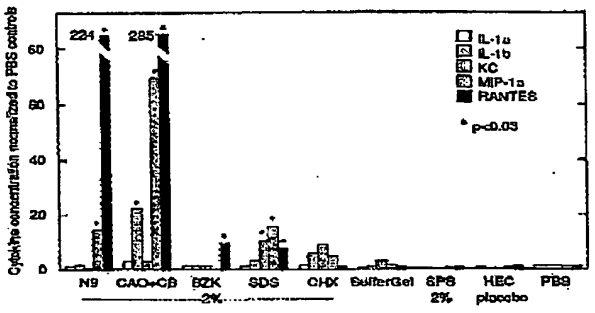

Figure 6
Inflammatory cytokines 12 hours after a single exposure to candidate microbicides. Geometric means for each agent are shown normalized by the results in PBS controls as follows, in ng/ml of lavage fluid: IL-1α, 16; IL-1β, 50; KC, 42; MIP-1α, 20; RANTES, 3. Thus N9 increased the concentration of RANTES 224-fold, from 3 to 672 ng/ml in lavage fluid. (The lavage procedure is estimated to have diluted vaginal secretions by 10–50-fold.) (CAO+CB) = 1%CAO+1%CB = 2% detergent.

reported that both N9 and SDS caused the same to happen in the mouse vagina, and we confirmed their findings for N9 and macrophages in this study. Milligan et al. also reported that two non-detergent candidate microbicides based on sulfated and sulfonated polymers, T-PSS and PRO 2000, did not cause significant increases in vaginal leukocytes even after repeated doses. Thus leukocyte entry into the lumen is a toxic effect that correlates in this mouse model with increased HSV susceptibility. However, unlike changes in epithelial thickness, the time-course of leukocyte entry does not correlate well with the increases in susceptibility: During the initial increase in susceptibility there was no significant increase in the numbers of leukocytes in the vagina, and at 6 hours, when the number of macrophages had markedly increased, HSV susceptibility returned essentially to normal. At 18 hours, HSV susceptibility again returned to normal but high numbers of both macrophages and neutrophils persist for many more hours [8].

Colposcopy

High-magnification colposcopy surprisingly failed to distinguish between mice exposed 12 hours earlier to N9 from controls treated with PBS. This result does not argue against performing colposcopic exams, but suggests instead that this method may not be adequate to detect certain toxic effects that cause major increases in susceptibility to HSV.

Inflammatory cytokines

Inflammatory cytokines are now being investigated during Phase I trials of vaginal microbicide candidates, not only as sensitive markers for epithelial damage, but also because they provide insight into important biological changes that may occur in the vagina as a result of microbicide administration. For example, increased chemokine concentrations correlate with an influx of immune and inflammatory cells into the vaginal mucosa. These cells can play a role in host immune defence, but CD4+ immune cells are also target cells for the HIV-1 virus, and their increased numbers could promote HIV-1 infection. Likewise, increased concentrations of Il-1 can activate NF-KB, an intracellular transcription signal that leads to the production of other inflammatory cytokines, and can promote HIV-1 shedding through activation of the HIV-1 LTR.

The results of the present study lend further support for screening candidate microbicides to insure they do not cause significant increases in inflammatory cytokines. The results in mice suggest that several different cytokines, and durations of exposure, should be investigated since no single cytokine served as a reliable predictor of significant toxicity at the time points chosen for this study. For example, although the detergents all caused a significant increase in RANTES, they did so to markedly different degrees at the 12-hour time point (N9 increased RANTES ~30-fold more than did SDS). Moreover, the major differences in cytokines released, as shown in Fig. 6, do not correlate with the almost *uniform* increase in susceptibility caused by all five detergents as shown in Fig. 3. Note also that in the mouse, even though a single dose of N9 markedly increased susceptibility to HSV it did not cause a detectable release of Il-6 and only insignificant trends occurred in Il-1α, Il-1β, and KC(Il-8), further emphasizing the lack of obvious correlation between cytokine release and HSV susceptibility. In the rabbit toxicity model Fichorova et al [10] found that N9, BZK, and SDS caused marked, and markedly different, increases in Il-6, IL-1β, and IL-8 when observed 24 hours after the first, second, and third daily application. In humans [38] a single application of N9 did not cause a detectable increase in any of the soluble mediators observed in lavage samples obtained after 12, 36, and 60 hours: IL-1α, IL-1β, IL-8, IL-1_{ra}, TNF-RI, TNF-RII (but at 12 hours, there was a significant decrease in SLPI). However, after 3 daily exposures, several mediators increased significantly: IL-1α and IL-1β (at 12, 36, and 60 hours after the last application), IL-8 (at 36 hours), MIP-1β (at 60 hours), TNF-RII (at 36 hours), neutrophil elastase (at 12 and 36 hours), and SLPI significantly decreased (at 12, 36 and 60 hours).

Human columnar endocervical epithelial cells are more sensitive to toxic effects of N-9 in vitro and release more

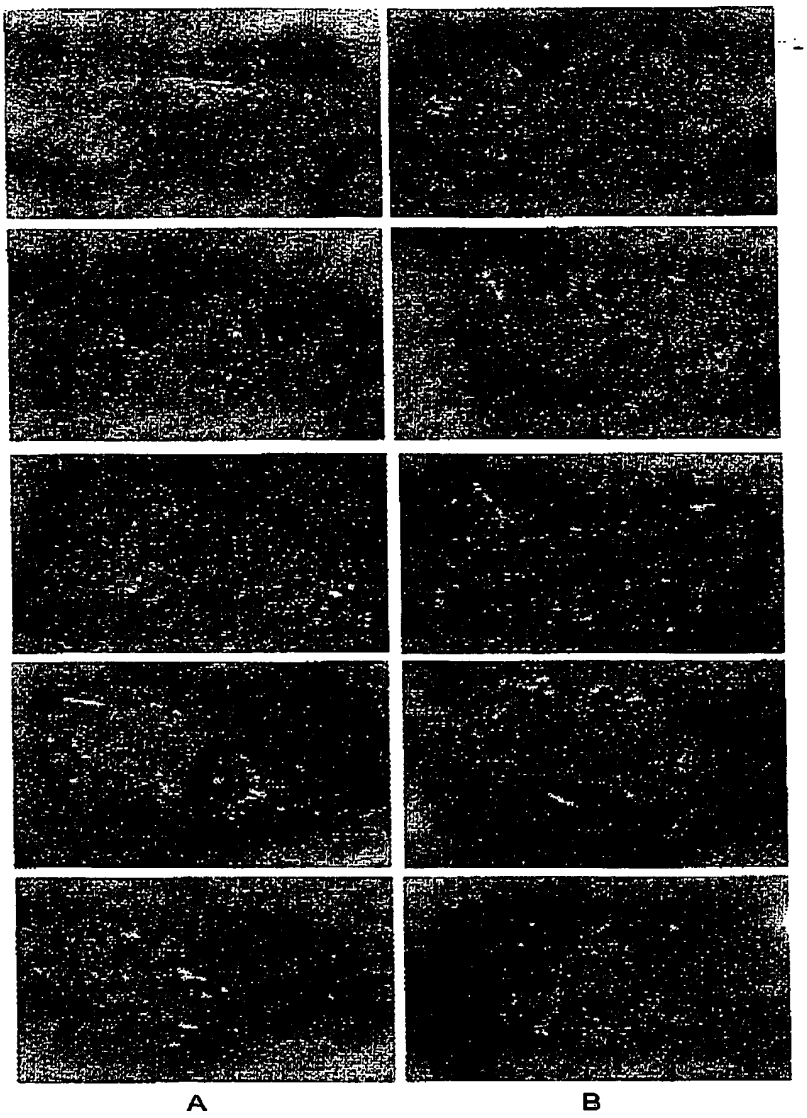

Figure 7
Colposcopic images of mouse vaginas 12 hours after a single exposure to 2% N9, or PBS. The colposcope (Zeiss OPMI I-SH) was set at maximum magnification (31×) and focused on the vaginal area surrounding the cervix. Column A, 2% N9, column B, PBS controls.

proinflammatory cytokines when damaged than do vaginal epithelial cells[10]. Similarly, in Swiss-Webster mice, the cervical columnar epithelium is more sensitive to the toxic effects of detergents than stratified squamous vaginal epithelium [5,6]. A major difference between vaginal lavage samples obtained from humans and the progestin-treated mice in the present study is the relatively small surface of columnar epithelium in humans that contributes to the lavage sample. Thus lavage samples from progestin-treated mice in which the entire vaginal epithelium is covered with living cells may provide a more sensitive measure of the effects of candidate microbicides on epithelia that are not protected by layers of squamous cells. This may explain why the increases in cytokines caused be single applications of the detergents were readily detectable in the mouse but not in humans.

Finally, our study also highlights the need to test microbicide agents and formulations for inhibitory effects on cytokine detection. SPS may have produced false negative readings in the cytokine test because it had a strong inhibitory effect on cytokine detection in the Bioplex assy.

Limitations of this study

Even though the mechanism of infection and pathogenesis of genital HSV is very similar in both humans and mice, there is still uncertainty predicting results in humans based on results in mice.

Pre-treatment with progestin (Depo-Provera) markedly increases HSV-2 susceptibility in mice, but it has not been documented whether it has this effect in humans. However, epithelia in both species that are not protected by a squamous layer are likely to be more susceptible to HSV.

Effects of formulation were not examined in this study, and different formulations may have different effects on toxicity (and efficacy).

Toxic effects of repeated exposures were not examined in this study. It is apparent that a single exposure to detergents and CHX caused significant toxicity, but other candidate microbicides may not cause significant toxicity unless applied repeatedly.

Results in this mouse/HSV model may not predict the effect of microbicides on human transmission of HIV, since there are significant differences in the mechanisms of transmission. In view of the 20–30 fold increase in HSV susceptibility a single dose of detergent causes in the mouse vagina, and the >100 fold increase in susceptibility N9 causes in the mouse rectum [20], it may be advisable to develop a primate model that directly detects whether a candidate microbicide increases susceptibility to SIV or SHIV since the surrogates now being monitored in animal models and Phase I trials may not reveal serious alterations in susceptibility.

Conclusion

1. The mouse model for vaginal transmission of HSV-2 can be used to detect toxicities caused by candidate microbicides that increase susceptibility to infection.

2. Detergents applied at a minimally protective dose caused rapid, prolonged, and major increases in vaginal HSV-2 susceptibility.

3. SPS and BufferGel are examples of candidate microbicides that protect against HSV infections without causing susceptibility-increasing toxic effects in this model.

4. In screening for toxic effects of microbicides in Phase I safety trials, it may be important to monitor a broad range of inflammatory cytokines vs time, and exfoliation of columnar epithelium.

Competing interests

RAC and TRM hold equity in ReProtect, Inc., which is developing BufferGel as a spermicidal microbicide. Authors declare there are no other competing interests.

Authors' contributions

RAC and TRM initiated the study and participated in the design and analysis throughout. RAC drafted the manuscript, and all authors read, helped revise, and approved the final manuscript. TH helped develop the procedures used in the mouse HSV model, and performed most of the experiments using this model. XXW performed some of the susceptibility tests that determined the time-course of susceptibility, and designed and performed the histology, macrophage entry, and colposcopy experiments, RA helped develop and perform the acute toxicity "dead cell" assay, and the inflammatory cytokine assays were performed in DJA's laboratory.

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Evaluations of Unformulated and Formulated Dendrimer-Based Microbicide Candidates in Mouse and Guinea Pig Models of Genital Herpes

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Prevention of sexually transmitted infections is a priority in developed and developing countries. One approach to prevention is the use of topical microbicides, and one promising approach is the use of dendrimers, highly branched macromolecules synthesized from a polyfunctional core. Three new dendrimer products developed to provide stable and cost-efficient microbicides were initially evaluated in vitro for anti-herpes simplex virus activity and then in vivo by using a mouse model of genital herpes. From these experiments one product, SPL7013, was chosen for further evaluation to define the dose and duration of protection. Unformulated SPL7013 provided significant protection from genital herpes disease and infection at concentrations as low as 1 mg/ml and for at least 1 h following topical (intravaginal) administration of 10 mg/ml. This compound was then formulated into three vehicles and further evaluated in mouse and guinea pig models of genital herpes infection. In the murine evaluations each of the formulations provided significant protection at concentrations of 10 and 50 mg/ml. Formulated compounds provided protection for at least 1 h at a concentration of 10 mg/ml. From these experiments formulation 2V was chosen for dose ranging experiments using the guinea pig model of genital herpes. The guinea pig evaluations suggested that doses of 30 to 50 mg/ml were required for optimal protection. From these studies a lead compound and formulation (2V of SPL7013) was chosen for ongoing evaluations in primate models of simian immunodeficiency virus and *Chlamydia trachomatis* infection.

The spread of sexually transmitted infections (STIs) continues to grow at an alarming rate. In the United States more than 12 million people are infected with STIs every year, accounting for 5 of the 10 most commonly reported infectious diseases (4). Globally the incidence of human immunodeficiency virus (HIV) infection continues to grow, with the most recent data from the United Nations showing that 40 million people worldwide are HIV positive. Similarly, infections with herpes simplex virus type 2 (HSV-2) continue to increase around the world at an alarming rate despite the availability of effective antivirals (11). Seroprevalence data suggest that >45 million patients are infected in the United States at this time, with projections for even further increases (5, 6). The high percentage of women infected with both HSV-2 and HIV is of particular concern. Because genital herpes can lead to an increased risk of HIV infections, prevention of genital herpesvirus infections may also impact the spread of HIV (5). Vaccines for STIs remain an important goal for reduction of the their spread; however, HIV vaccines remain an elusive goal, while the prospects for vaccines for other STIs, including genital herpesvirus and human papillomavirus infection, are more encouraging (8, 12).

Microbicides, defined as a chemical entity that can prevent or reduce transmission of STIs when applied to the vagina or rectum, represent an intriguing approach to the prevention of STIs. Most microbicide candidates act by disrupting the cell membrane or envelope of the pathogen (for example, detergents such as nonoxynol-9), by blocking receptor-ligand interactions (for example, sulfated compounds, such as PRO 2000), or by modifying the vaginal environment (for example, pH buffering agents such as Buffer-gel) (reviewed in references 10, 14, and 15).

Dendrimers are a relatively new class of macromolecules characterized by highly branched three-dimensional architectures that offer an alternative to polyanionic polymers. They are assembled in a precise stepwise manner, and this controlled synthesis allows the assembly of highly defined "nanoobjects," in contrast to the heterogenous nature of traditional polymer-based materials. Therefore, we applied this technology to prepare defined macromolecular polyanions that would retain good levels of activity against the early stages of viral infection and have optimum physical properties (i.e., low systemic absorption, water solubility, ease of formulation, etc.) for microbicide development. In vitro and in vivo studies on a selection of these compounds have been reported previously and showed that they are potent inhibitors of a range of sexually transmitted diseases. Several compounds inhibited the replication of HIV type 1 with a 50% effective concentration (EC_{50}) of <1 μg/ml (19), while members of this same class of dendrimer were also effective in vitro against HSV-1 and HSV-2 (3). These compounds appeared to inhibit the early stages of virus replication although there was some evidence of effects on the late stages of viral replication (17, 19). In addi-

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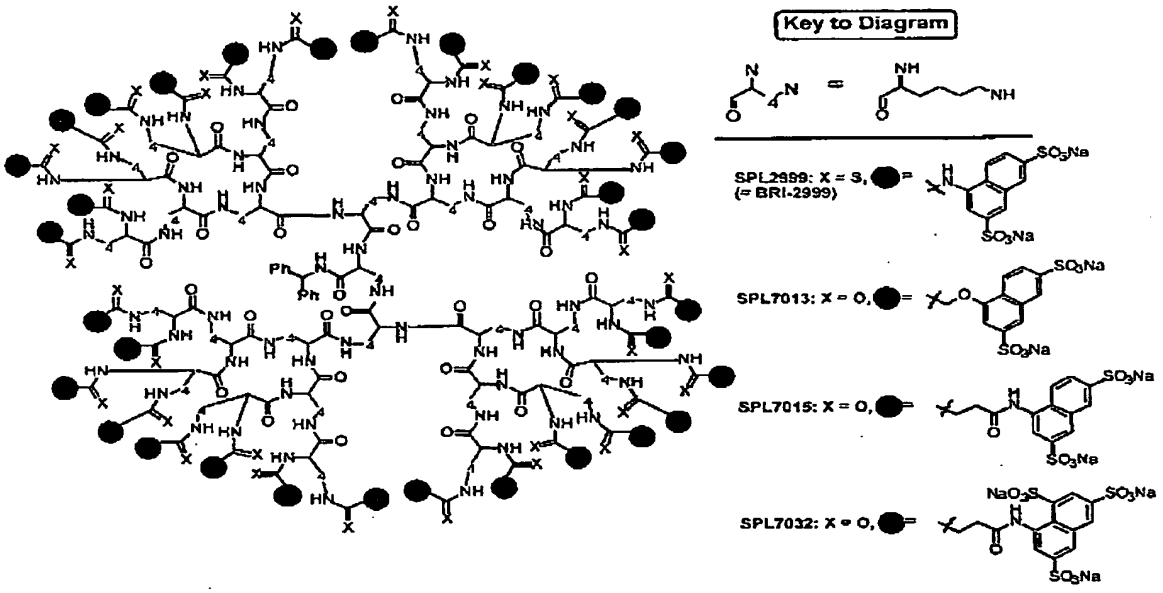

FIG. 1. Molecular structures of SPL2999, SPL7013, SPL7015, and SPL7032. The lysine dendrimer generations are built out from a central core (boldface), through a series of layers or generations. The outer sulfonic acid surface is attached via thiourea (SPL2992) or amide (SPL7013, SPL7015, and SPL7032) linkages.

tion, the compounds were nontoxic to the cells up to the highest concentration tested, 100 μg/ml (3).

Recently, dendrimers dissolved in saline (i.e., unformulated) were used in in vivo evaluations of activity in a mouse model of genital herpes (3). These early dendrimer-based microbicide candidates were prepared by assembling aromatic-sulfonic acid or aromatic-carboxylic acid units to the outer surface of lysine- or polyamidoamine-based dendrimers via a thiourea linkage (for example, see compound SPL2999 in Fig. 1, which has previously been referred to as BRI-2999) (3, 7). Since that time we have further refined the dendrimer architecture in order to produce compounds in a Good Laboratory Practice/Current Good Manufacturing Practice environment with the required stability and cost-of-production profile necessary for a microbicide candidate. This work resulted in the identification of three new microbicide candidates, SPL7013, SPL7015, and SPL7032 (Fig. 1), which are all prepared from the same lysine dendrimer and where the earlier thiourea linkage has been replaced by a more stable amide bond.

This paper reports on the in vivo evaluation of these stable, expanded-spectrum dendrimers when used in mouse and guinea pig models of HSV-2-induced genital herpesvirus infection. In addition we report the in vivo activity of three prototype formulations of the selected development candidate, SPL7013. This formulated preparation is currently undergoing in vivo evaluations in macaques for efficacy against simian/human immunodeficiency virus and *Chlamydia trachomatis*.

MATERIALS AND METHODS

Viruses. HSV-2 strain G was used for in vitro assays (7), while HSV-2 strain 186 was used for mouse inoculations (3) and strain MS was used for guinea pig inoculations (2). All viruses were prepared as described previously (2, 3, 7).

Dendrimers. A powder form of the dendrimers was supplied by Starpharma (Prahran, Australia). Solutions (1 to 100 mg/ml) were prepared in phosphate-buffered saline (PBS) and stored at room temperature.

Formulations. Formulation development work on SPL7013 was performed at the Center for Pharmaceutical Science and Technology at the University of Kentucky. Methylparaben (NF), propylparaben (NF), edetate disodium dihydrate (EDTA; USP), propylene glycol (USP), glycerin (USP), and sodium hydroxide (NF) were obtained from Spectrum Quality Products, Inc. (New Brunswick, N.J.). Carbopol 971P (NF) was purchased from BF Goodrich Specialty Chemicals (Cleveland, Ohio).

A range of excipients were initially investigated, but ultimately research focused on carbopol-based aqueous gels due to their mucoadhesive properties and their use in vaginal products and other microbicide formulations. As shown in Table 1, three different gel prototype SPL7013 formulations (1V, 2V, and 3V) were developed containing 5, 1, 0.1, and 0% (wt/wt) SPL7013. The prototypes differed only in the final percentage (weight per weight) of propylene glycol and glycerin. All of the gel formulations contained a final carbopol 971P (NF) concentration of 4.75 to 5% (wt/wt) depending on the final concentration of SPL7013 in the gel. Carbopol 971P (NF) is a cross-linked acrylic acid listed in the *USP Monograph* as Carbomer 941. After initial evaluations, 1 to 5% SPL7013-containing formulation 2V was also prepared.

The gels were made with a Caframo (Wiarton, Ontario, Canada) stirrer, model BDC-1850, by adding the required amount of purified water, EDTA, and then propylene glycol and glycerin. Next, carbopol 971P (NF) was slowly added to avoid clumping, and the formed gel was mixed until the polymer was fully hydrated (~1 h). The pH was adjusted to 4.5 with 2 N NaOH, and then methylparaben and propylparaben were added and mixed until dissolved. The gel was then made to weight with purified water, and the final pH was adjusted to 4.5

TABLE 1. Excipients for three prototype vaginal microbicide placebo gels

Excipient	Prototype gel	Amt (wt/wt) (%)	Function
Water for injection, USP	All	100[a]	Vehicle
Methylparaben, NF	All	0.18	Antimicrobial preservative
Propylparaben, NF	All	0.02	Antimicrobial preservative
EDTA, USP	All	0.1	Antioxidant
Carbopol 971, NF	All	5.0	Gelling agent
Propylene glycol, USP	1V	5.0	Emollient
	2V	1.0	
	3V	0	
Glycerin, USP	1V	5.0	Emollient
	2V	1.0	
	3V	0	
2 N NaOH to pH 4.5	All	9.0	pH-adjusting agent

[a] Make up to 100%.

with either 2 N NaOH or 1 N HCl. The viscosity of all gels was measured with a cone and plate rheometer, model RVDV III+ (Brookfield Engineering; Middleboro, Mass.), at 25°C for 5 min at 1.7 rpm by using spindle CPE-52. For all gels, the viscosity of the formulations was in the range of 30,000 to 43,000 cP under the conditions described above.

Each formulation was assessed for toxicity in a 5-day rabbit vaginal-irritation study prior to evaluation. Each of the placebo prototype gels and those gels containing 1 and 5% (wt/wt) SPL7013 elicited the same level of minimal irritation in a 5-day repeat dose rabbit vaginal model (data not shown).

In vitro evaluations. Confluent Vero cell monolayers in six-well plates were incubated in duplicate with different concentrations of dendrimers ranging from 0.01 to 30 μg/ml at 37°C for 1 h. One hundred PFU of HSV-2 strain G were then added to the cells, and the samples were incubated at 37°C for 1 h. After the inoculum was removed, the cells were washed with PBS and overlaid with 0.5% methylcellulose for a plaque assay. After 2 days the monolayers were fixed with 10% formalin and stained with 0.5% crystal violet as previously described (7). EC_{50} values were calculated with the Statview computer program.

The cytotoxicity of the compounds was also evaluated by using Vero cells following incubation with various concentrations of the test compounds for 2 days and examination using the neutral-red uptake assay as previously described (7).

Animal models. All animal protocols were approved by the Cincinnati Children's Hospital Animal Use and Care Committee. All procedures complied with the relevant federal and institutional policies.

Mouse model of genital HSV-2 infection. As previously described (2, 3) female Swiss Webster mice weighing 18 to 21 g (Harlan, Indianapolis, Ind.) were given 0.1 ml of a suspension containing 3 mg of medroxyprogesterone acetate (Upjohn Pharmacia, Kalamazoo, Mich.) by subcutaneous injection 7 days and 1 day prior to challenge to increase susceptibility to vaginal HSV infection. Animals were then anesthetized, and the vaginas were swabbed with a calcium alginate swab prior to intravaginal inoculation of the formulated or unformulated dendrimer or placebo in a volume of 15 μl. Following various defined intervals the animals were then challenged with 15 μl of a suspension containing 10^4 PFU of HSV-2 strain 186 applied intravaginally without removal of the preceding treatment material. Vaginal swabs were collected from all animals on day 2 after inoculation and stored frozen (−80°C) until assayed for the presence of virus on susceptible rabbit kidney cells. Animals were then monitored daily for 21 days for evidence of herpetic disease, including hair loss and erythema around the perineum, chronic urinary incontinence, hind-limb paralysis, and death. For the purpose of these studies animals that did not develop symptoms were defined as infected if virus was isolated from the vaginal swab specimens collected on day 2 after inoculation (2, 3).

Guinea pig model of genital herpes. As previously described, Hartley guinea pigs weighing 275 to 300 g (Charles River Breeding Laboratory, Wilmington, Mass.) were treated intravaginally with 200 μl of formulated dendrimer or placebo, followed by intravaginal inoculation with 200 μl of a suspension containing 10^5 PFU of HSV-2 strain MS without removal of the preceding treatment material (2). Vaginal swabs were obtained on days 1 and 2 postinoculation and stored frozen (−80°) until assayed for the presence of virus on susceptible rabbit kidney cells. For the purpose of these studies animals that did not develop symptoms were defined as infected if virus was isolated from the vaginal swab specimens collected on day 1 or 2 after inoculation (2).

Statistics. Incidence data were compared by Fisher's exact test. All comparisons were two-sided. No corrections were made for multiple comparisons.

TABLE 2. Antiviral activity of dendrimers against HSV-2 determined by plaque reduction assay[a]

Dendrimer	EC_{50} (μg/ml)	CC_{50} (μg/ml)[b]
SPL7013	0.6[c]	>1,000
SPL7015	0.5	>1,000
SPL7032	0.7	>1,000

[a] Evaluations were repeated five times for SPL7013, twice for SPL7015, and once for SPL7032.
[b] CC_{50}, cytotoxic concentration.
[c] The standard deviation was 0.03 μg/ml.

TABLE 3. Evaluation of three expanded-spectrum dendrimer products against genital herpes in mice[b]

Treatment	Concn (mg/ml)	Fraction (%) of animals protected against:	
		Disease	Infection
SPL7013	100	11/11 (100)[a]	11/11 (100)[a]
SPL7015	100	9/12 (75)[a]	8/12 (67)[a]
SPL7032	100	11/12 (92)[a]	11/12 (92)[a]
PBS		0/12 (0)	0/12(0)

[a] $P < 0.005$ versus PBS.
[b] Mice were treated 20 s prior to challenge.

RESULTS

In vitro. All three compounds had similar in vitro activities against HSV-2, with no evidence of toxicity even at the highest concentration tested, 1,000 μg/ml (Table 2).

Animal models. (i) Unformulated dendrimers. In the initial experiment 10% solutions of SPL7013, -7015, and -7032 (Fig. 1) were evaluated in mice. Significant protection by each compound against disease and infection was observed (Table 3) when the time from treatment to virus challenge was minimal (20 s). From this and similar comparisons and because of the ease of manufacturing, cost, and stability, SPL7013 was chosen for further development.

In the subsequent experiments either the effect of drug concentration or the duration of protection was evaluated. As seen in Table 4 compound SPL7013 provided significant protection at concentrations as low as 1 mg/ml when the time from treat-

TABLE 4. Effect of concentration on protection from genital herpes by dendrimer SPL7013 in mice[c]

Treatment	Concn (mg/ml)	Fraction (%) of animals protected against:	
		Disease	Infection
SPL7013	100	12/12 (100)[a]	12/12 (100)[a]
SPL7013	10	11/12 (92)[a]	10/12 (83)[a]
SPL7013	1	8/12 (67)[b]	6/12 (50)[b]
PBS		0/12 (0)	0/12(0)

[a] $P < 0.001$ versus PBS.
[b] $P < 0.05$ versus PBS.
[c] Mice were treated 20 s prior to challenge.

TABLE 5. Duration of protection by dendrimer SPL7013 against genital herpes in mice

Treatment	Concn (mg/ml)	Time (min) treated prior to challenge	Fraction (%) of animals protected against:	
			Disease	Infection
SPL7013	10	5	14/16 (88)[a]	14/16 (88)[a]
SPL7013	10	30	13/16 (81)[a]	12/16 (75)[a]
PBS		5	0/16 (0)	0/16 (0)
SPL7013	10	60	5/15 (33)[b]	4/15 (27)
PBS		5	0/15 (0)	0/15 (0)

[a] $P < 0.001$ versus PBS.
[b] $P < 0.05$ versus PBS.

ment to challenge was minimal. As seen in Table 5 this compound, at a concentration of 10 mg/ml, provided significant protection from disease for at least 1 h following administration.

(ii) Formulated dendrimers. Three different formulations of dendrimer SPL7013 were then prepared at the University of Kentucky at concentrations of 1 and 5% (Table 1). In the initial experiment each formulation of 1% SPL7013 was evaluated in the mouse model of genital HSV infection. As seen in Table 6 each formulation provided significant protection when administered 5 min prior to intravaginal challenge. Note also that the placebo formulation provided some protection against disease but not infection. This is most likely due to the buffering effect of the formulation in maintaining the acid pH of the vagina. In the subsequent experiment the duration of protection out to 30 min after treatment with the 5% concentration of each formulation was evaluated. Again, significant protection against infection and disease was provided by each formulation (Table 7). The 2V formulation was chosen for further evaluation and was shown to provide significant protection at a concentration of 1% for 30 min in two experiments and for at least 1 h after application in the one experiment where this was evaluated (Table 8).

The 2V formulation of SPL7013 was further evaluated in the guinea pig model of genital herpes because this model, it is felt, better mimics human disease (13). In the initial experiments 1 to 5% concentrations of SPL7013 in formulation 2V were applied 5 min prior to virus challenge. As seen in Table 9,

TABLE 6. Evaluation of three 1% formulations of dendrimer SPL7013 against genital herpes in mice[e]

Treatment	Concn (mg/ml)	Fraction (%) of animals protected against:	
		Disease	Infection
SPL7013 formulation 1V	10	11/16 (69)[a,c]	11/16 (69)[a,d]
Placebo 1V		3/16 (19)	1/16 (6)
SPL7013 formulation 2V	10	12/16 (75)[a]	12/16 (75)[a,c]
Placebo 2V		7/16 (44)[b]	4/16 (25)
SPL7013 formulation 3V	10	13/15 (87)[a,c]	12/15 (80)[a,c]
Placebo 3V		6/16 (38)[b]	4/16 (25)
PBS		1/16 (6)	1/16 (6)

[a] $P < 0.001$ versus PBS.
[b] $P < 0.05$ versus PBS.
[c] $P < 0.05$ versus placebo.
[d] $P < 0.001$ versus placebo.
[e] Mice were treated 5 min prior to challenge.

TABLE 7. Evaluation of three 5% formulations of dendrimer SPL7013 against genital herpes in mice

Treatment	Concn (mg/ml)	Time (min) treated prior to challenge	Fraction (%) of animals protected against:	
			Disease	Infection
SPL7013 formulation 1V	50	30	10/16 (63)[a]	10/16 (63)[a]
SPL7013 formulation 2V	50	30	15/16 (94)[a]	15/16 (94)[a]
SPL7013 formulation 3V	50	30	16/16 (100)[a]	16/16 (100)[a]
SPL7013 formulation 1V	50	5	14/16 (88)[a]	13/16 (81)
SPL7013 formulation 2V	50	5	15/16 (94)[a]	15/16 (94)[a]
SPL7013 formulation 3V	50	5	15/16 (94)[a]	15/16 (94)[a]
PBS		5	0/16 (0)	0/16 (0)

[a] $P < 0.001$ versus PBS.

protection appeared to be dose dependant, with increased protection at 3 to 5% concentrations. The experiment was repeated to determine if the decreased activity of the 30-mg/ml dose would be confirmed. Repeat experiments showed that protection with this concentration was not diminished in comparison to that with lesser concentrations. Thus, the second experiment confirmed the high protection rates provided by the 3 and 5% concentrations and were consistent with dose-dependant activity. The activity seen in the placebo recipients in the first experiment is consistent with that observed in some of the mouse studies (Table 6) with formulation 2V.

DISCUSSION

The continued HIV epidemic and ongoing increases in the prevalence of genital HSV-2 and other STIs underscore the need for a safe effective user-controlled strategy to prevent these infections. Microbicides offer one such strategy. Because of the lack of efficacy and possible deleterious effects of N-9, a nonionic surfactant that disrupts lipid membranes, such as viral envelopes (16, 18), compounds that inhibit binding, such as polyanions, rather than acting as detergents are receiving increased attention (reviewed in references 10, 14, and 15). One potential drawback of the polyanions in clinical development as topical microbicides is that they are mixtures of compounds. PRO 2000 (1, 9), for example, is a polymer mixture of between 4 and 6 kDa, and Carraguard contains various carbohydrates with various levels of sulfation. In contrast, SPL7013 has been characterized by mass spectrometry, capillary electrophoresis,

TABLE 8. Evaluation of duration of protection of 1% 2V formulation of SPL7013 against genital herpes in mice

Treatment	Concn (mg/ml)	Time (min) treated prior to challenge	Fraction (%) of animals protected against:	
			Disease	Infection
SPL7013 formulation 2V	10	5	8/15 (53)[a]	8/15 (53)[a]
SPL7013 formulation 2V	10	30	9/15 (60)[a]	8/15 (53)[a]
SPL7013 formulation 2V	10	60	6/15 (40)[b]	6/15 (40)[b]
PBS		5	0/15 (0)	0/15 (0)
SPL7013 formulation 2V	10	5	8/12 (67)[a]	8/12 (67)[a]
SPL7013 formulation 2V	10	30	8/12 (67)[a]	8/12 (67)[a]
PBS		5	0/12 (0)	0/12 (0)

[a] $P < 0.01$ versus PBS.
[b] $P < 0.05$ versus PBS.

TABLE 9. Evaluation of protection of different concentrations of formulation 2V of SPL7013 against genital herpes in guinea pigs[d]

Treatment	Concn (mg/ml)	Fraction (%) of animals protected against:	
		Disease	Infection
SPL7013 formulation 2V	10	7/15 (47)	5/15 (33)
SPL7013 formulation 2V	20	10/15 (67)	9/15 (60)[a]
SPL7013 formulation 2V	30	7/15 (47)	7/15 (47)[a]
SPL7013 formulation 2V	40	11/15 (73)[a]	10/15 (67)[a]
SPL7013 formulation 2V	50	12/15 (80)[a]	11/15 (73)[a]
Placebo gel		10/15 (67)	8/15 (53)[a]
PBS		4/15 (27)	1/15 (7)
SPL7013 formulation 2V	30	16/18 (89)[b,c]	15/18 (83)[b,c]
SPL7013 formulation 2V	50	17/18 (94)[b,c]	16/18 (89)[b,c]
Placebo gel		3/18 (17)	2/18 (11)
PBS		4/18 (22)	3/18 (17)

[a] $P < 0.05$ versus PBS.
[b] $P < 0.001$ versus PBS.
[c] $P < 0.001$ versus placebo.
[d] Animals were treated 5 min prior to challenge.

and high-pressure liquid chromatography, and in-process controls have been developed to tightly control the synthesis. As a result SPL7013 has entered full preclinical development as a topical microbicide.

In this paper we have shown that dendrimer SPL7013 provides protection from infection and disease in the mouse model of genital herpes even at concentrations as low as 1 mg/ml and for at least 1 h after administration. Similarly, after formulation this candidate microbicide remained active when used in the guinea pig model of genital herpes. Thus, despite the increased size, vaginal vault area, and higher dose of virus used in the guinea pig model, the high activity was maintained. Note also that, although good activity was maintained after formulation, there was no obvious advantage to the formulated product. Continuing evaluations are aimed at determining if the formulated products have advantages either in the duration of protection or dose effects in both the mouse and guinea pig models. Further, whether there might be advantages in larger animals, such as the primates that are currently being evaluated and humans, remains to be determined. The goal of the formulation should be to increase the spread of the material so it is more effective, increase the time it is present in the vaginal cavity through mucoadhesive or other properties to increase the duration of protection, or provide additional activity, for example, by maintaining the vaginal pH.

From both the mouse and guinea pig evaluations it appears that concentrations of 3% or higher of the formulated product may be necessary for optimal protection. Because of the encouraging results with this formulated dendrimer in the experiments presented here, evaluations in monkey models of simian/human immunodeficiency virus and chlamydia are ongoing. Dendrimer SPL7013 is one of the leading candidates to fulfill the difficult requirements of a microbicide to be safe yet active against a number of STIs.

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Murine cytomegalovirus resistant to antivirals has genetic correlates with human cytomegalovirus

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Human cytomegalovirus (HCMV) resistance to antivirals is a significant clinical problem. Murine cytomegalovirus (MCMV) infection of mice is a well-described animal model for *in vivo* studies of CMV pathogenesis, although the mechanisms of MCMV antiviral susceptibility need elucidation. Mutants resistant to nucleoside analogues aciclovir, adefovir, cidofovir, ganciclovir, penciclovir and valaciclovir, and the pyrophosphate analogue foscarnet were generated by *in vitro* passage of MCMV (Smith) in increasing concentrations of antiviral. All MCMV antiviral resistant mutants contained DNA polymerase mutations identical or similar to HCMV DNA polymerase mutations known to confer antiviral resistance. Mapping of the mutations onto an MCMV DNA polymerase three-dimensional model generated using the *Thermococcus gorgonarius* Tgo polymerase crystal structure showed that the DNA polymerase mutations potentially confer resistance through changes in regions surrounding a catalytic aspartate triad. The ganciclovir-, penciclovir- and valaciclovir-resistant isolates also contained mutations within MCMV M97 identical or similar to recognized GCV-resistant mutations of HCMV UL97 protein kinase, and demonstrated cross-resistance to antivirals of the same class. This strongly suggests that MCMV M97 has a similar role to HCMV UL97 in the phosphorylation of nucleoside analogue antivirals. All MCMV mutants demonstrated replication-impaired phenotypes, with the lowest titre and plaque size observed for isolates containing mutations in both DNA polymerase and M97. These findings indicate DNA polymerase and protein kinase regions of potential importance for antiviral susceptibility and replication. The similarities between MCMV and HCMV mutations that arise under antiviral selective pressure increase the utility of MCMV as a model for *in vivo* studies of CMV antiviral resistance.

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INTRODUCTION

Human cytomegalovirus (HCMV) is an important pathogen of immunocompromised individuals, and the development of antiviral-resistant HCMV strains is an increasing hindrance to the successful treatment and prevention of CMV-related illness. Animal models are necessary for *in vivo* studies of CMV antiviral susceptibility and resistance due to the species-specificity of HCMV that restricts permissive replication to humans and human cell lines. Murine cytomegalovirus (MCMV) infection in mice is an excellent model that has been extensively studied with respect to pathology and immunology (Hudson, 1979; Koffron *et al.*, 1998; Lagenaur *et al.*, 1994; Yuhasz *et al.*, 1994). The entire MCMV genome has also been sequenced, and shares many regions of similarity with HCMV (Rawlinson *et al.*, 1993, 1996).

HCMV and MCMV are inhibited by antivirals that target the viral DNA polymerase, including nucleoside analogues aciclovir (ACV), adefovir (ADV), cidofovir (CDV), ganciclovir (GCV), penciclovir (PCV), the ACV pro-drug valaciclovir (VCV) and the pyrophosphate analogue foscarnet (PFA) (De Clercq, 2001; Rawlinson, 2001; Smee *et al.*, 1995). HCMV and MCMV are inhibited by similar concentrations of these antivirals *in vitro*, except MCMV exhibits increased sensitivity to ACV (Boyd *et al.*, 1993, Chrisp & Clissold, 1991; Cole & Balfour, 1987; Rawlinson *et al.*, 1997; Smee

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et al., 1995; Xiong *et al.*, 1997a, b). The nucleoside analogues ACV, ADV, CDV, GCV and PCV require intracellular phosphorylation prior to incorporation into replicating viral DNA by the HCMV DNA polymerase (De Clercq, 2001). In HCMV-infected cells, ACV, GCV and PCV are initially monophosphorylated by the HCMV UL97 protein kinase, and further phosphorylated to the triphosphate form by cellular enzymes (Biron *et al.*, 1985; Littler *et al.*, 1992; Sullivan *et al.*, 1992; Talarico *et al.*, 1999; Zimmerman *et al.*, 1997). ADV and CDV are monophosphorylated and do not require activation by viral proteins prior to cellular conversion to their triphosphate forms (Xiong *et al.*, 1997a, b).

HCMV strains resistant to ADV, CDV, PFA or GCV can develop through mutations of the DNA polymerase gene (encoded by UL54), which can confer multi-drug resistance depending on the mutation position (Chou, 1999; Chou *et al.*, 2003; Erice, 1999). HCMV UL97 protein kinase mutations are most often described as responsible for the development of GCV-resistance in HCMV isolates, although earlier studies omitted assessment of DNA polymerase mutations (Chou, 1999; Erice, 1999). Although currently not documented in clinical isolates, mutations of HCMV UL97 protein kinase can confer resistance to ACV and PCV (Talarico *et al.*, 1999; Zimmerman *et al.*, 1997), with potential implications for the use of VCV prophylaxis (Feinberg *et al.*, 1997; Lowance *et al.*, 1999).

MCMV M54 and M97 are positional, sequence and primary structure homologues of HCMV DNA polymerase and UL97 protein kinase (Elliott *et al.*, 1991; Rawlinson *et al.*, 1993, 1996). The MCMV homologues retain DNA polymerase and protein kinase domain regions important for protein function (Elliott *et al.*, 1991; Rawlinson *et al.*, 1997). No other protein kinase homologues are known to be encoded by MCMV (Rawlinson *et al.*, 1996). Phosphorylation assays have failed to show detectable levels of ACV- and GCV-phosphorylation either in MCMV-infected cells or by a recombinant M97 protein, despite MCMV susceptibility to ACV and GCV *in vitro* (Burns *et al.*, 1981; Ochiai *et al.*, 1992; Wagner *et al.*, 2000). However, while HCMV UL97 substituted for MCMV M97 enhances GCV phosphorylation by recombinant MCMV, HCMV UL97 does not complement the function of MCMV M97 with respect to virus replication (Wagner *et al.*, 2000). Additional correlative information is therefore necessary to determine the complementarities of HCMV and MCMV antiviral susceptibility and virus replication.

In order to further analyse CMV replication and antiviral susceptibility, we have generated a series of MCMV mutants resistant to ACV, ADV, CDV, GCV, PCV, PFA and VCV and characterized the genetic mutations associated with the resultant changes in phenotype. Regions of MCMV DNA polymerase and the putative protein kinase (pM97) important for MCMV antiviral susceptibility and virus replication have been identified. The mutations of these MCMV antiviral-resistant strains demonstrate remarkable similarity to mutations known to confer antiviral resistance to HCMV isolates defined from infected patients, and correlate with DNA polymerase and protein kinase regions of functional importance.

METHODS

Antivirals. Reagent grade ACV and VCV were kindly provided by GlaxoWellcome (UK), ADV and CDV by Gilead Sciences (USA), GCV by Roche Pharmaceuticals (Australia), PCV by SmithKline Beecham Pharmaceuticals (UK) and PFA by Astra Pharmaceuticals (Australia). Antivirals were resuspended in pyrogen-free water (Baxter) to a final concentration of 10 μM (except PFA, which was resuspended to a final concentration of 50 μM), filter-sterilized and stored in aliquots at −20 °C until use.

MCMV strains. MCMV laboratory strain Smith was obtained from ATCC. The virulent laboratory strain K181 and nine wild-type MCMV isolates (G3A, G4, K17A, K17G, N1, W2, W5, W8 and W9) were kindly provided by Professor Geoffrey Shellam from the Department of Microbiology, University of Western Australia, Perth, Australia. Viral titres were quantified in primary mouse embryo fibroblasts (MEFs) by standard plaque assays, as described previously (Scott *et al.*, 2000).

Generation of antiviral-resistant MCMV isolates. MCMV antiviral resistant mutants (ACVres, ADVres, CDVres, GCVres, PCVres, PFAres and VCVres) were generated by continuous passaging of MCMV laboratory strain Smith in MEFs in increasing concentrations of each antiviral. Mutants were selected against both ACV and the ACV pro-drug VCV, to compare the resistance mutations generated by antiviral agents that differed in bioavailability. Initially, Smith strain was cultured in 25 cm^2 flasks containing minimal essential media (MEM) + 2 % fetal bovine serum (FBS) and 0·1 μM of antiviral. Virus was passaged to new MEF cultures after 100 % cytopathic effect (CPE) was observed, or one week of culture where CPE was less than 100 %, and antiviral concentration increased twofold at each passage. At higher antiviral concentrations, cultures were carried out in six-well plates, where plates were centrifuged at 600 *g* for 30 min to enhance virus infectivity. Passaging of virus continued to the highest concentration of antiviral where virus continued to be isolated without cellular toxicity (Table 1).

Each antiviral-resistant mutant was plaque purified a total of five times by culturing serial 1 : 10 dilutions of virus and sterile selection of single plaques using a pipette set at 20 μl. Single plaques were amplified by culture in six-well plates containing MEFs between purification rounds. At the final round of plaque purification, mutant virus was cultured in the presence of media without antiviral and cell-associated virus harvested and stored in MEM + 10 % FBS at −80 °C.

Plaque reduction assays (PRA). PRA was carried out in triplicate using 50 p.f.u. per well of virus as described previously (Rawlinson *et al.*, 1997), except that plates were centrifuged at 600 *g* for 30 min following inoculation of virus. The concentration range for each antiviral was as follows: 0·47–120 μM ACV (increased to 1·825–480 μM for analysis of the VCV-resistant mutant), 0·93–240 μM ADV, 0·0625–16 μM CDV, 0·625–160 μM GCV, 1·25–320 μM PCV, 6·25–1800 μM PFA and 1·825–480 μM VCV. The 50 and 90 % inhibitory concentrations (IC_{50} and IC_{90}) were calculated by linear regression from plots of percentage reduction in plaque numbers at each antiviral concentration against log drug concentrations. Resistance was defined as a greater than twofold increase in IC_{50} and IC_{90} values compared with parent (Smith) strain.

M54 and M97 DNA PCR. M54 and M97 PCR was carried out on DNA extracted from MCMV-infected MEFs as described previously (Scott *et al.*, 2002). The entire M54 gene was amplified in four

Table 1. Antiviral susceptibilities of wild-type MCMV parent strain (Smith) and corresponding MCMV antiviral-resistant mutants

Antiviral	Mutant selection* (μM)	Parent IC_{50}† (μM)	Mutant IC_{50}† (μM)	Increase‡
ACV	60	1·2±0·1	26·0±2·5	21·7
ADV	60	0·5±0·1	135·1±70·1	270·2
CDV	8	0·2±0·2	7·0±3·9	35
GCV	80	6·5±2·0	51·8±18·4	6·7
PCV	160	8·1±1·7	22·2±4·8	2·7
PFA	800	80·9±14·7	439·8±65·1	5·4
VCV	240	2·3±0·6	279·1±140·2	121·3

*Final antiviral concentration for isolation of the antiviral resistant mutant.
†IC_{50}, 50 % inhibitory concentration (μM) of antiviral.
‡Indicates fold increase of mutant IC_{50} from that of the parent strain (Smith).

overlapping segments (M54A, M54B, M54C and M54D) of approximately 800 to 900 bp. M54A was amplified using primers M54.T8 (5′-ATCAATCGAGATAAGGGAGGG-3′) and M54.B7 (5′-TGGAGCCCTCGCCGCGAACGT-3′), M54B using primers M54.T6 (5′-CATAAGGGAAACGAACTACTT-3′) and M54.B5 (5′-CGACGGACAGCAGATCAGGAT-3′), M54C using primers M54.T4 (5′-GTTGGGCAAGATCATGTCCCG-3′) and M54.B3 (5′-AGAGCAGAGCAACTCGCCGTT-3′), and M54D using primers M54.T2 (5′-AAGGACAGGCAACGGTAGAAC-3′) and M54.B1 (5′-CTCCGATTTCGAGTACTGACG-3′). The entire M97 gene was amplified by PCR in three overlapping segments (M97A, M97B and M97C) of approximately 800 to 900 bp, using primers M97.T1 (5′-TCGGATCACTCTGGTGTGTG-3′) and M97.B7 (5′-AGCCCGCCGCTAGAGGAAGC-3′) for M97A, M97.T3 (5′-ATTCCGTGTCGGTCGCCCGGT-3′) and M97.B4 (5′-GGCCAGGCCCGCGTAGTGCC-3′) for M97B, and M97.T5 (5′-AGCGTCTACTGCAACATCCT-3′) and M97.B1 (5′-GACCGTCGTCGCATATCTGG-3′) for M97C. Each reaction consisted of 50 mM KCl, 10 mM Tris/HCl pH 9·0, 0·1 % Triton-X-100, 5 mM $MgCl_2$, 0·25 mM dNTP, 0·4 μM forward and reverse primer, 1 U *Taq* DNA polymerase (Promega) and 2 μl DNA template in a total reaction volume of 50 μl. Reaction conditions consisted of an initial denaturation step at 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min.

M54 and M97 sequencing. PCR products were purified as described previously using polyethylene glycol (PEG) 8000 (Scott *et al.*, 2002), and sequenced using ABI Prism BigDye terminator chemistry (Applied Biosystems). Forward and reverse PCR primers were used for sequencing, as well as internal forward and reverse primers specific for each PCR product. The entire M54 and M97 gene sequences were assembled by comparison to Smith consensus sequences (Rawlinson *et al.*, 1996), translated using Alltrans and whole protein sequences for each gene aligned using CLUSTALW (Thompson *et al.*, 1994). The HCMV UL54 DNA polymerase and UL97 protein kinase sequences from laboratory strain AD169 (Chee *et al.*, 1990) and homologues of other herpesviruses were also included in the alignments for comparison. The DNA polymerase sequences of the nine wild-type MCMV strains and seven MCMV antiviral-resistant mutants were submitted to GenBank and have the accession numbers AY529137–AY529146 and AY529127–AY529133, respectively. The pM97 sequences from GCVres, PCVres and VCVres were also submitted to GenBank and have the accession numbers AY529134–AY529136.

DNA polymerase modelling. Protein structures with similar sequence to HCMV and MCMV DNA polymerase were identified by a BLAST search of the protein database (www.rscb.org/pdb) and these homologous polymerase proteins were aligned using CLUSTALX (Thompson *et al.*, 1997). This alignment included the bacteriophage RB69 DNA polymerase that has previously been used as a model for the analysis of herpes simplex virus and CMV DNA polymerase resistance mutations (Huang *et al.*, 1999; Chou *et al.*, 2003). An MCMV DNA polymerase model was built using the structure of the closest matched template of known three-dimensional structure, the Tgo polymerase from *Thermococcus gorgonarius* (PDB code 1TGO) (Hopfner *et al.*, 1999) with the help of COMPOSER as contained in Sybyl6.92 (Tripos). The model was subjected to energy minimization to optimize the geometry and remove steric overlap in the structure and Verify3D (Luthy *et al.*, 1992) was used to evaluate the quality of the model.

Growth kinetic assays. Growth kinetic assays comparing the wild-type parent strain (Smith) to each of the antiviral-resistant mutants were carried out in triplicate. Virus was inoculated at an m.o.i. of 0·01 p.f.u. per cell onto MEFs with centrifugal enhancement and cultured for 4 days. Cell-free (media) and cell-associated virus (trypsinized cells) were harvested and stored at −80 °C. Cell-free and cell-associated virus titres were quantified by calculation of tissue culture infective dose (TCID) in 96-well plates containing confluent MEFs. The number of infected cells per plaque for each MCMV strain was also counted after 4 days of culture to determine focus expansion and plaque size measured following photography. These analyses were performed as a blind trial.

RESULTS

Antiviral susceptibilities of the MCMV antiviral-resistant mutants

All seven MCMV antiviral-resistant mutants (ACVres, ADVres, CDVres, GCVres, PCVres, PFAres and VCVres) were isolated in antiviral concentrations that exceeded the IC_{50} and IC_{90} values of MCMV-sensitive strains (Rawlinson *et al.*, 1997; Smee *et al.*, 1995). PRA confirmed the MCMV mutants had significant increases in IC_{50} and IC_{90} values compared with those of the parent (Smith) strain (Table 1). The increases in antiviral inhibitory concentrations ranged from 2·7-fold for the PCVres mutant to 270·2-fold for the ADVres mutant compared with the parent (Smith) strain (Table 1).

Mutants generated against nucleoside analogues that require phosphorylation (ACV, GCV, PCV and VCV) also demonstrated cross-resistance to other antivirals within this group

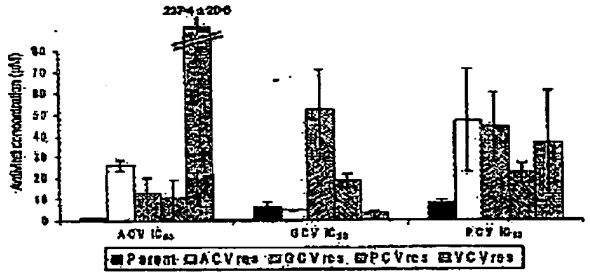

Fig. 1. MCMV mutants resistant to ACV (ACVres), GCV (GCVres), PCV (PCVres) and VCV (VCVres) are cross-resistant to other nucleoside analogue antivirals. The IC_{50} of ACV, GCV and PCV are shown with error bars for parent (Smith) strain and MCMV mutants resistant to ACV (ACVres), GCV (GCVres), PCV (PCVres) and VCV (VCVres). Large standard deviations were observed for PCV IC_{50} compared with other antivirals.

(Fig. 1). The GCVres and PCVres mutants demonstrated moderate levels of cross-resistance to ACV, with approximately 10-fold increases in IC_{50} values compared with parent (Smith) strain (Fig. 1), and 12- and 15-fold increases in IC_{90} values, respectively (results not shown). As expected, resistance to ACV was also observed for the MCMV mutant generated against VCV (Fig. 1). The level of resistance that VCVres demonstrated to ACV was very high, requiring inhibitory concentrations of ACV that were 200-fold that of the parent (Smith) strain, and 10-fold that of the ACVres mutant (Fig. 1). Cross-resistance to GCV was only observed with the PCVres mutant, and not ACVres or VCVres. All mutants tested (ACVres, GCVres and VCVres) demonstrated cross-resistance to PCV (Fig. 1).

MCMV DNA polymerase mutations associated with resistant phenotype

All seven antiviral-resistant mutants contained mutations of MCMV DNA polymerase (Table 2 and Fig. 2). These mutations occurred in regions of MCMV DNA polymerase that were invariant in nine sequenced wild-type (antiviral sensitive) strains (AY529137–AY529146) and mutations were verified by repeat amplification and sequencing reactions using a second set of different primers. No MCMV DNA polymerase mutations were found outside of codons 360–870 corresponding to the DNA polymerase domain region (Fig. 2), identified from alignment with HCMV sequences (Chou *et al.*, 1999). Half of the MCMV DNA polymerase mutations (4/8) occurred within or in close proximity to DNA polymerase domain III (Table 2 and Fig. 2).

The ACVres, ADVres and VCVres mutants contained identical amino acid mutations (P826R) between DNA polymerase functional domains I and VII (Table 2 and

Table 2. MCMV M54 (DNA polymerase) and M97 (protein kinase) mutations associated with antiviral resistance correlate with HCMV resistance mutations

MCMV mutant	Cross-resistance	DNA polymerase resistance mutations			Protein kinase resistance mutations		
		M54 (DNApol) mutation	DNA polymerase domain	HCMV correlates (resistance)*	M97 (PK) mutation	Protein kinase domain	HCMV correlates (resistance)*
ACVres	PCV	P826R	Between I and VII	V955I (VCV)[1]†	Nil	-	-
ADVres	ND	P826R	Between I and VII	V955I (VCV)[1]†	ND	-	-
CDVres	ND	L467I	δ-region C	L501I (CDV, GCV)[2]	ND	-	-
		D709N§	III	K805Q (CDV)[2]			
		V717L	III	V812L (GCV, CDV, PFA, ADV)[3]			
GCVres	ACV, PCV	L678V	VI	V781I (PFA, GCV)[3,4]	P479L	Between VIII and IX	-
		P734S	III	A834P (GCV, CDV, PFA)[3]			
PCVres	ACV, GCV	T753A‡	III	-	M395V	VIb	M460V (GCV)[5]
PFAres	ND	A679V	VI	V781I (PFA, GCV)[3,4]	Nil	-	-
VCVres	ACV, PCV	P826R	Between I and VII	V955I (VCV)[1]†	T393M	VIb	M460V (GCV)[5]

*HCMV resistance mutations homologous or occurring in similar regions to the MCMV mutations ([1] Scott *et al.*, 2004; [2] Chiby *et al.*, 1998a; [3] Chiby *et al.*, 1998b; [4] Jabs *et al.*, 2001; [5] Chou *et al.*, 1995). MCMV resistance mutations with identical HCMV mutations are indicated in bold.

†These HCMV mutations have not been verified by marker transfer experiments.

‡MCMV mutations D709N and T753A reside 1 aa from the N terminus and 3 aa from the C terminus of DNA polymerase domain III, respectively.

ND, Not done.

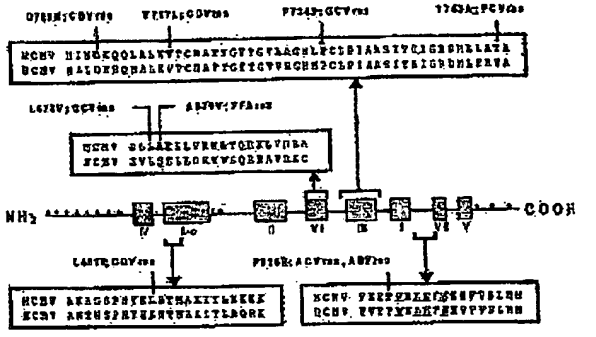

Fig. 2. DNA polymerase mutations associated with antiviral resistance. Fragments of the MCMV Smith sequence (Rawlinson *et al.*, 1996) and HCMV AD169 sequence (Chee *et al.*, 1990) are shown as the reference consensus sequences for selected DNA polymerase domains. Mutations associated with MCMV antiviral resistance are shown in bold, in relation to the domain or region in which they occur and their relationship with HCMV resistance mutations is shown in italics. A mutation found in a temperature-sensitive mutant (Ihara *et al.*, 1994) is shown underlined, and the bdExE metal binding motif between DNA polymerase domains I and VII (Hopfner *et al.*, 1999) is shown in underlined italics. Regions of variation observed in antiviral-sensitive (wild-type) strains are indicated by an asterisk (*). Variation between domain VII and the C terminus occurred in only 5/11 of isolates.

Fig. 2). These mutants were isolated at different time points, and the ADVres mutation verified by repeat plaque purification of the mutant from original stocks and repeat sequencing. The CDVres mutant contained one mutation corresponding to DNA polymerase δ-region C (L467I) and two additional mutations at DNA polymerase domain III (D709N and V717L) (Table 2 and Fig. 2). The CDVres D709N mutation was located one codon outside the N-terminal end of DNA polymerase domain III, whereas mutation V717L lay in the centre of domain III. Two mutations were found in the GCVres mutant within DNA polymerase domains VI (L678V) and III (P734S) (Table 2 and Fig. 2). The only DNA polymerase mutation of PCVres (T753A) was located three codons outside of the C-terminal end of domain III (Fig. 2). A mutation within DNA polymerase domain VI was detected in the PFAres mutant (A679V), one residue downstream of the GCVres domain VI mutation (L678V) (Table 2 and Fig. 2).

Alignment of mutant MCMV and HCMV DNA polymerase sequences indicated two of the CDVres mutations (L467I and V717L) were identical in location and amino acid change to HCMV mutations (L501I and V812L) associated with antiviral resistance (Cihlar *et al.*, 1998a, b) (Table 2 and Fig. 2). The location of the other MCMV resistance mutations, except T753A of PCVres, also correlated with regions of HCMV DNA polymerase associated with antiviral resistance (Cihlar *et al.*, 1998a, b; Scott *et al.*, 2004). This included the third mutation of CDVres (D709N), located one codon upstream of the HCMV mutation K805Q associated with CDV resistance and the DNA polymerase domain III N terminus (Cihlar *et al.*, 1998a).

MCMV DNA polymerase three-dimensional structure

An MCMV DNA polymerase model generated using the *T. gorgonarius* Tgo polymerase crystal structure had good Verify3D scores for the majority of the protein (Fig. 3a). *T. gorgonarius* Tgo polymerase demonstrated greater similarity to HCMV and MCMV DNA polymerase than bacteriophage RB69 DNA polymerase, which has previously been used as a model for the study of herpes simplex virus and CMV antiviral resistance mutations (Huang *et al.*, 1999; Chou *et al.*, 2003). The catalytic domain regions of MCMV DNA polymerase exhibited the best Verify3D scores, and the N-terminal region of the protein model had the lowest score (Fig. 3a). A small number of insertions in the MCMV sequence relative to the template protein were not amenable to modelling and were removed from the model during alignment. These regions were predominantly within the N-terminal or extreme C-terminal region of MCMV DNA polymerase.

The MCMV DNA polymerase mutations associated with antiviral resistance in this study were analysed using the model (Fig. 3b). Only subtle perturbations in the MCMV DNA polymerase model were observed on alteration of the residues associated with antiviral resistance. However, most of the resistance mutations were located in the model surrounding an MCMV aspartate triad (D624, D791 and D793) homologous to the catalytic aspartate triad (D717, D910 and D912) of *T. gorgonarius* Tgo polymerase, a motif important for nucleotidyl transfer and metal binding (Rodriguez *et al.*, 2000).

M97 protein mutations associated with resistant phenotype

Mutants resistant to antivirals that require phosphorylation (PCVres, GCVres and VCVres) contained mutations of pM97 not present in MCMV antiviral-sensitive strains, in addition to the DNA polymerase mutations described above (Rawlinson *et al.*, 1997) (Table 2 and Fig. 4). Mutations of pM97 were not detected in the ACVres mutant, suggesting the P826R M54 (DNApol) mutation described above is solely responsible for the antiviral resistance of this isolate (Table 2 and Fig. 4). No pM97 mutations were detected in PFAres, demonstrating non-specific M97 mutations did not result from passaging of virus.

Regions of pM97 corresponding to protein kinase functional domains were defined by alignment with HCMV UL97 protein kinase, herpes simplex virus UL13 and other protein kinase sequences (Chee *et al.*, 1989; Hanks & Quinn,

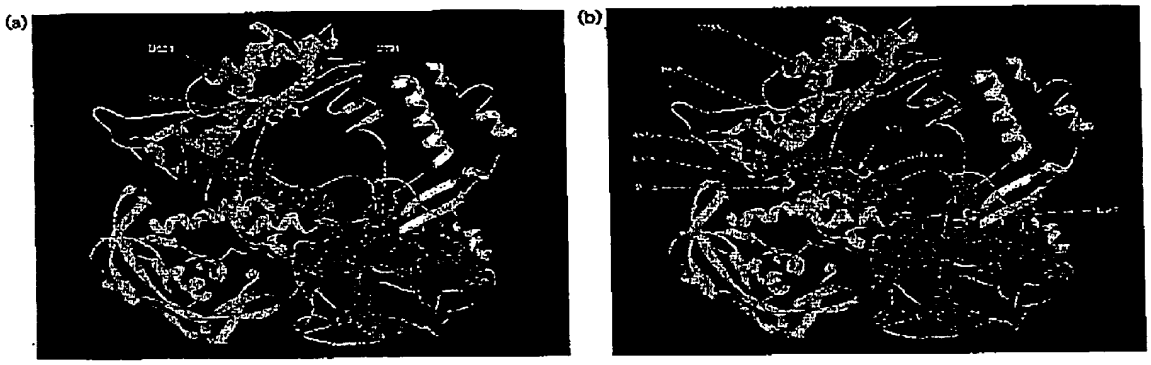

Fig. 3. MCMV DNA polymerase three-dimensional model. (a) A ribbon representation of MCMV DNA polymerase coloured according to region (yellow, N-terminal region; blue, exonuclease region; white, thumb region; green, palm region; red, finger regions) with the catalytic aspartate triad (D624, D791 and D793) drawn in bond representation coloured by atom type. (b) Ribbon model of MCMV DNA polymerase with residues identified as giving rise to drug resistance drawn in bond representation coloured by atom type.

1991). The pM97 mutations of the PCVres and VCVres mutants (M395V and T393M, respectively) occurred within regions corresponding to the protein kinase domain VIb

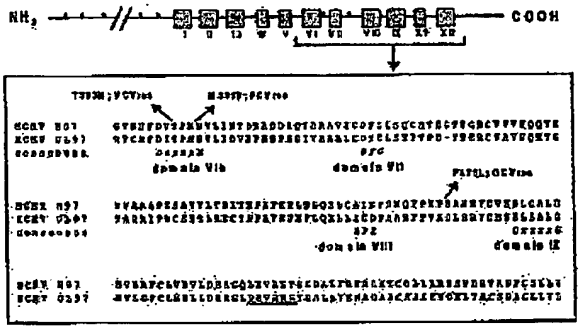

Fig. 4. MCMV pM97 and HCMV UL97 protein kinase antiviral resistance mutations. Alignment of the protein sequences of MCMV Smith M97 (Rawlinson *et al.*, 1996) and HCMV AD169 UL97 (Chee *et al.*, 1990) with MCMV resistance mutations identified in this study are indicated by bold font, and HCMV GCV resistance mutations from previous studies are indicated in italics (Chou, 1999; Erice, 1999). The putative domain IX consensus motif described by Chee *et al.* (1989) is underlined, although alignment with MCMV M97 suggests domain IX of UL97 is further towards the N terminus as indicated by consensus motif *DxxxxG* as position 488–493 of MCMV and 552–557 of HCMV. Regions of variation in wild-type antiviral-sensitive strains are indicated by an asterisk (*) (Rawlinson *et al.*, 1997).

conserved motif (*DxxxxN*) (Table 2 and Fig. 4). The M97 mutation of GCVres (P479L) was located between consensus motifs for protein kinase domains VIII (*APE*) and IX (*DxxxxG*) (Table 2 and Fig. 4). Alignment of the MCMV pM97 sequences with UL97 protein kinase sequences from HCMV antiviral-resistant isolates indicated the pM97 domain VIb mutation of PCVres was homologous to a mutation (M460V) that confers HCMV resistance to GCV, with the pM97 mutation of VCVres only two codons upstream of this site (Fig. 4) (Chou *et al.*, 1995).

Replication impairment of the MCMV antiviral-resistant mutants

All MCMV antiviral-resistant mutants were impaired in replication ability, producing smaller plaque sizes and 3- to 363-fold less cell-associated and cell-free virus titres than the parent (Smith) strain (Fig. 5). The most reduction in replication ability was observed with the VCVres mutant that contained single DNA polymerase and pM97 mutations (Fig. 5). The replication impairment of VCVres was greater than that observed for the ACVres and ADVres mutants (all containing identical DNA polymerase mutations), indicating the additional pM97 mutation of VCVres contributed to the overall decrease in replication ability of this isolate (Fig. 5 and Table 2). More than one mutation in MCMV DNA polymerase (the CDVres and GCVres mutants) or mutations in both DNA polymerase and pM97 (the GCVres, PCVres and VCVres mutants) tended to have a greater impact on replication ability than single DNA polymerase mutations. The PFAres mutant that contained a single DNA polymerase mutation corresponding to domain VI was the least replication-impaired mutant (three- to fivefold reduction in $TCID_{50}$) (Fig. 5). Only a moderate reduction in plaque size was observed for the PFAres mutant at day 4, and

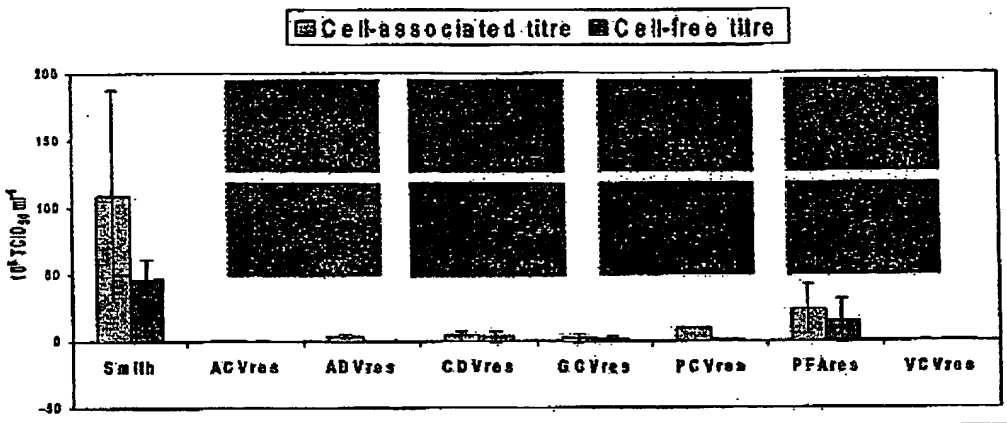

Fig. 5. Growth kinetics of MCMV parent (Smith) and antiviral resistant mutant strains in culture. The $TCID_{50}$ of cell-associated and cell-free MCMV parent (Smith) strain and antiviral resistant mutants after 4 days of culture in MEFs (initially inoculated at an m.o.i. of 0·01) are graphed. Insets are representative plaques produced by the parent (Smith) and antiviral resistant mutants after 7 days in culture with plaque size ratings (+, smallest; +++++, largest).

by day 7 the PFAres plaque sizes were equivalent to those observed for parent Smith strain (Fig. 5).

DISCUSSION

The antiviral-resistant and replication-impaired phenotypes of the seven MCMV mutants were associated with M54 DNA polymerase and the M97 putative protein kinase mutations that developed *in vitro* under selective pressure from antivirals. The involvement of the MCMV mutations in reduced antiviral susceptibility and replication ability is further supported by their correlation with HCMV mutations associated with antiviral resistance and their detection within protein regions of functional importance. In turn, the MCMV mutations associated with antiviral resistance and replication impairment have suggested important regions of MCMV DNA polymerase and pM97 that indicate functional elements of the HCMV and cellular homologues of these proteins.

The MCMV DNA polymerase mutation P826R between domains I and VII of the ACVres, ADVres and VCVres mutants was identical in position but different in substitution to a mutation (P826A) found in an ACV-resistant MCMV mutant generated in a separate study (Minematsu *et al.*, 2001). Furthermore, this MCMV mutation correlates with an HCMV DNA polymerase mutation (V955I) we have recently identified in a patient who developed an HCMV-related illness despite receiving VCV prophylaxis (Scott *et al.*, 2004). This suggests the region between DNA polymerase domains I and VII is potentially associated with CMV ACV susceptibility, which is enhanced for MCMV compared with HCMV (Cole & Balfour, 1987; Rawlinson *et al.*, 1997; Smee *et al.*, 1995). Domain I is the most conserved functional domain of DNA polymerases and is of major importance in substrate binding, polymerase activity and virus replication (Huang *et al.*, 1999; Ye & Huang, 1993). Domain VII is involved in polymerase activity and recognition of ACV (Hwang *et al.*, 1992; Ye & Huang, 1993). These two important domains are maintained as β-sheets in a herpesvirus DNA polymerase model (Huang *et al.*, 1999), and β-sheet turns are predominantly associated with proline (P) residues (Stryer, 1988). This suggests the antiviral resistance and replication impairment observed for ACVres, ADVres and VCVres might have resulted from altered interaction of DNA polymerase domains I and VII with nucleoside analogues and natural substrate.

The similarity observed between MCMV and HCMV mutations associated with antiviral resistance suggests MCMV antiviral-resistance and replication-impairment is potentially conferred via similar mechanisms as HCMV. Two mutations of CDVres (the δ-region C mutation L467I and domain III mutation V717L) were identical in location and amino acid substitution to HCMV DNA polymerase mutations that confer resistance to CDV and GCV (Cihlar *et al.*, 1998a, b; Lurain *et al.*, 1992). The L467I mutation is identical in position to a δ-region C mutation of HCMV DNA polymerase (L501F) that produces enhanced exonuclease activity (Kariya *et al.*, 2000), and presumably more efficient removal of incorporated inhibitor. The third CDVres mutation (D709N) aligned adjacent to the N-terminal codon of HCMV DNA polymerase domain III (K805), which when substituted with glutamine (Q) confers resistance to CDV (Cihlar *et al.*, 1998a). The D709N mutation is also congruent with domain III mutations of

HCMV DNA polymerase that interfere with DNA template binding (Ye & Huang, 1993). The P734S mutation of the GCVres mutant is in close proximity to an HCMV DNA polymerase mutation we have detected in an HCMV strain isolated from a patient demonstrating clinical resistance to GCV, PFA and CDV (Scott *et al.*, 2004). The T753A mutation of the PCVres mutant did not correlate with antiviral-resistant mutations of HCMV, but was in close proximity to ACV-resistant mutations of herpes simplex virus and varicella-zoster virus that result in decreased substrate binding (Huang *et al.*, 1999; Schmit & Boivin, 1999; Visse *et al.*, 1999). The A679V mutation of DNA polymerase domain VI was the only genetic alteration identified in the PFAres mutant, and corresponds in location with mutations of HCMV and other herpesvirus DNA polymerases associated with PFA-resistance (Cihlar *et al.*, 1998a; Schmit & Boivin, 1999). DNA polymerase domains II and VI are the only two functional domains of HCMV DNA polymerase associated with PFA-resistance (Cihlar *et al.*, 1998a). The GCVres mutant also contained a mutation (L678V) within MCMV DNA polymerase domain VI, and an association between this domain and HCMV GCV resistance has been suggested but not proven (Jabs *et al.*, 2001).

The conservative nature of almost all the resistance mutations identified in MCMV polymerase suggests that the mutations alter antiviral susceptibility of the polymerase via subtle perturbations of the protein. This is supported by analysis of the MCMV antiviral resistance mutations using the MCMV DNA polymerase model generated using the backbone of *T. gorgonarius* Tgo polymerase crystal structure (Hopfner *et al.*, 1999). Most of the resistance mutations in MCMV CDVres, GCVres, PCVres and PFAres are clustered in domains III and VI and surround a catalytic triad formed by conserved aspartic acid residues D624 (domain II), D791 and D793 (both in domain I). Analyses of *Thermococcus* sp. polymerases have demonstrated the importance of this catalytic aspartate triad for nucleotidyl transfer and Mg^{2+} binding (Rodriguez *et al.*, 2000). Therefore, the MCMV mutations potentially alter the local structure and hence orientation of the three aspartates in the triad, reducing the polymerase efficiency. This resulting reduction in polymerase efficiency would give the exonuclease domain an improved chance to remove any defective DNA strands containing antiviral nucleoside analogues, allowing the viral polymerase to complete replication successfully. A further potential mechanism of resistance involving disruption of an Mn^{2+}- and Zn^{2+}-binding site known to associate with the catalytic aspartate triad (Hopfner *et al.*, 1999) is also suggested by the P826R mutation present in MCMV ACVres, ADVres and VCVres given this residue's proximity to this metal-binding region. The position of L467I at the end of a helix in the nuclease domain suggests that this mutation may alter the processivity of the nuclease activity and potentially have a concomitant effect on the incorporation of inhibitors. Detailed study of the enzymology of the MCMV DNA polymerase will shed further light on the mechanism of action of these antiviral resistance mutations.

Interestingly, the two MCMV mutants generated against ACV (one against the ACV pro-drug VCV) produced identical DNA polymerase mutations (P826R), but the VCVres mutant also contained an additional mutation in M97. It is not known whether MEFs contain the specific dipeptide transporters responsible for the increased bioavailability of VCV in intestinal cells (Han *et al.*, 1998; Landowski *et al.*, 2003), nor VCV hydrolase required for cleavage of L-valine from VCV to produce ACV (Burnette *et al.*, 1995). It is therefore unclear whether these mechanisms contributed to the additional M97 mutation observed in the VCVres mutant, and no evidence exists suggesting the frequency of additional resistance mutations is related to exposure to higher antiviral concentrations. However, similar to results observed here with MCMV VCVres, HCMV isolates containing mutations in both DNA polymerase and UL97 have increased resistance to GCV compared with isolates containing single UL97 mutations (Smith *et al.*, 1997). In HCMV isolates, high-level resistance related to the length of exposure to antiviral (Smith *et al.*, 1997), whereas ACVres and VCVres were exposed to antiviral for similar lengths of time. The differences observed here for ACVres and VCVres were therefore most likely the result of random selection, enhanced by the process of plaque purification for each mutant.

The detection of three separate pM97 mutations in MCMV mutants resistant to ACV, GCV and PCV suggest the involvement of the putative M97-protein kinase in MCMV susceptibility to nucleoside analogues that require phosphorylation by virally encoded enzymes. Two of these mutations (T393M and M395V of the MCMV VCVres and PCVres mutants, respectively) reside within a region of pM97 homologous to the protein kinase domain VIb consensus motif (*DxxxxN*) important for phosphotransfer and catalytic activity (Chee *et al.*, 1989; Hanks & Hunter, 1995). The M395V mutation of the PCVres mutant is identical in position and amino acid substitution to the UL97 protein kinase mutation (M460V) frequently detected in GCV-resistant HCMV isolates (reviewed by Chou, 1999; Erice, 1999). The MCMV GCVres mutation (P479L) did not correlate with HCMV UL97 protein kinase mutations associated with antiviral resistance, and cannot be directly attributed to the observed phenotypic changes of this mutant due to the presence of two additional DNA polymerase mutations. However, the GCVres P479L mutation is located midway between protein kinase domain VIII (*APE*) involved in peptide recognition, and the peptide substrate binding domain IX (*DxxxxG*), and is therefore positionally central to the protein kinase catalytic core (Hanks & Hunter, 1995). No mutations were detected in the pM97 sequence (KTCDAL) homologous with the UL97 protein kinase AACRAL motif that has a strong association with antiviral resistance in HCMV GCV-resistant strains (Chou, 1999; Erice, 1999; Sullivan *et al.*, 1993). Furthermore, the domain VIb mutation of MCMV VCVres did not confer cross-resistance to GCV despite being homologous to an HCMV UL97 protein kinase mutation that is common in

HCMV GCV-resistant isolates (Chou, 1999; Erice, 1999). This difference may account for the low levels of GCV phosphorylation by MCMV pM97 compared with HCMV UL97 (Wagner *et al.*, 2000). However, the low levels of phosphorylated GCV produced in MCMV-infected cells are sufficient for inhibition of MCMV replication, as shown in this study and others (Rawlinson *et al.*, 1997; Smee *et al.*, 1995; Wagner *et al.*, 2000), suggesting MCMV DNA polymerase has a high affinity for trace levels of phosphorylated nucleoside analogues produced by the M97 putative protein kinase (Wagner *et al.*, 2000).

MCMV and HCMV are similar in the DNA polymerase and protein kinase mutations that develop under antiviral selective pressure, the evidence suggesting potential involvement of the respective protein kinase homologues in antiviral susceptibility to nucleoside analogues, and the DNA polymerase and protein kinase regions that appear to be of functional importance. These similarities indicate a close relationship between MCMV and HCMV antiviral resistance and demonstrate the utility of MCMV as an animal model for CMV antiviral studies.

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